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<b>(21) International Application Number:</b> PCT/US96/20090 <b>(22) International Filing Date:</b> 13 December 1996 (13.12.96)  <b>(30) Priority Data:</b> 08/572,027 14 December 1995 (14.12.95) US  <b>(60) Parent Application or Grant</b> <b>(63) Related by Continuation</b> US 08/572,027 (CON) Filed on 14 December 1995 (14.12.95)  <b>(71) Applicant (for all designated States except US):</b> CARGILL, INCORPORATED [US/US]; 15407 McGinty Road West, Wayzata, MN 55391 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> DeBONTE, R., Lorin [US/US]; 3706 Rochdale Drive, Fort Collins, CO 80525 (US). FAN, Zhegong [US/US]; 4429 Monte Carlo Place, Fort Collins, CO 80525 (US). LOH, H.-T., Willie [US/US]; 5236 West Lake Nokomis Parkway, Minneapolis, MN 55417 (US).	<b>(74) Agent:</b> LUNDQUIST, C., Ronald; Fish & Richardson P.C., Suite 3300, 60 South 6th Street, Minneapolis, MN 55402 (US).  <b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>	
<b>(54) Title:</b> PLANTS HAVING MUTANT SEQUENCES THAT CONFER ALTERED FATTY ACID PROFILES  <b>(57) Abstract</b> <p>Seeds, plants and oils are provided having low FDA saturates; high oleic acid; low linoleic acid; high or low palmitic acid; low stearic acid; and low linoleic acid plus linolenic acid; and advantageous functional or nutritional properties. Plants are disclosed that contain a mutation in a delta-12 or delta-15 fatty acid desaturase gene. Preferred plants are rapeseed and sunflower plants. Plants carrying such mutant genes have altered fatty acid composition in seeds. In one embodiment, a plant contains a mutation in a region having the conserved motif His-Xaa-Xaa-Xaa-His, found in delta-12 and delta-15 fatty acid desaturases. A preferred motif has the sequence His-Glu-Cys-Gly-His. A preferred mutation in this motif has the amino acid sequence His-Lys-Cys-Gly-His. Nucleic acid fragments are disclosed that comprise a mutant delta-12 or delta-15 fatty acid desaturase gene sequence.</p>		

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5                    PLANTS HAVING MUTANT SEQUENCES THAT CONFER ALTERED  
                    FATTY ACID PROFILES

Technical Field

                    This invention relates to *Brassica* seeds and  
plants having mutant sequences which confer altered fatty  
acid profiles on the seed oil. More particularly, the  
10 invention relates to mutant delta-12 and delta-15 fatty  
acid desaturase sequences in such plants which confer  
such profiles.

Background of the Invention

                    Diets high in saturated fats increase low density  
15 lipoproteins (LDL) which mediate the deposition of  
cholesterol on blood vessels. High plasma levels of  
serum cholesterol are closely correlated with  
atherosclerosis and coronary heart disease (Conner et  
al., *Coronary Heart Disease: Prevention, Complications,*  
20 *and Treatment*, pp. 43-64, 1985). By producing oilseed  
*Brassica* varieties with reduced levels of individual and  
total saturated fats in the seed oil, oil-based food  
products which contain less saturated fats can be  
produced. Such products will benefit public health by  
25 reducing the incidence of atherosclerosis and coronary  
heart disease.

                    The dietary effects of monounsaturated fats have  
also been shown to have dramatic effects on health.  
Oleic acid, the only monounsaturated fat in most edible  
30 vegetable oils, lowers LDL as effectively as linoleic  
acid, but does not affect high density lipoproteins (HDL)  
levels (Mattson, F.H., *J. Am. Diet. Assoc.*, 89:387-391,  
1989; Mensink et al., *New England J. Med.*, 321:436-441,  
1989). Oleic acid is at least as effective in lowering  
35 plasma cholesterol as a diet low in fat and high in

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carbohydrates (Grundy, S.M., New England J. Med.,  
314:745-748, 1986; Mensink et al., New England J. Med.,  
321:436-441, 1989). In fact, a high oleic acid diet is  
preferable to low fat, high carbohydrate diets for  
5 diabetics (Garg et al., New England J. Med., 319:829-834,  
1988). Diets high in monounsaturated fats are also  
correlated with reduced systolic blood pressure (Williams  
et al., J. Am. Med. Assoc., 257:3251-3256, 1987).  
Epidemiological studies have demonstrated that the  
10 "Mediterranean" diet, which is high in fat and  
monounsaturates, is not associated with coronary heart  
disease (Keys, A., Circulation, 44(Suppl):1, 1970).

Many breeding studies have been conducted to  
improve the fatty acid profile of *Brassica* varieties.  
15 Pleines and Freidt, Fat Sci. Technol., 90(5), 167-171  
(1988) describe plant lines with reduced C<sub>18:3</sub> levels (2.5-  
5.8%) combined with high oleic content (73-79%). Rakow  
and McGregor, J. Amer. Oil Chem. Soc., 50, 400-403 (Oct.  
1973) discuss problems associated with selecting mutants  
20 for linoleic and linolenic acids. In. Can. J. Plant  
Sci., 68, 509-511 (Apr. 1988) Stellar summer rape  
producing seed oil with 3% linolenic acid and 28%  
linoleic acid is disclosed. Roy and Tarr, Z.  
Pflanzenzuchtg, 95(3), 201-209 (1985) teaches transfer of  
25 genes through an interspecific cross from *Brassica juncea*  
into *Brassica napus* resulting in a reconstituted line  
combining high linoleic with low linolenic acid content.  
Roy and Tarr, Plant Breeding, 98, 89-96 (1987) discuss  
prospects for development of *B. napus* L. having improved  
30 linolenic and linolenic acid content. European Patent  
application 323,751 published July 12, 1989 discloses  
seeds and oils having greater than 79% oleic acid  
combined with less than 3.5% linolenic acid. Canvin,  
Can. J. Botany, 43, 63-69 (1965) discusses the effect of

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temperature on the fatty acid composition of oils from several seed crops including rapeseed.

Mutations typically are induced with extremely high doses of radiation and/or chemical mutagens (Gaul, H. Radiation Botany (1964) 4:155-232). High dose levels which exceed LD50, and typically reach LD90, led to maximum achievable mutation rates. In mutation breeding of Brassica varieties high levels of chemical mutagens alone or combined with radiation have induced a limited number of fatty acid mutations (Rakow, G.Z. Pflanzenzuchtg (1973) 69:62-82). The low  $\alpha$ -linolenic acid mutation derived from the Rakow mutation breeding program did not have direct commercial application because of low seed yield. The first commercial cultivar using the low  $\alpha$ -linolenic acid mutation derived in 1973 was released in 1988 as the variety Stellar (Scarth, R. et al., Can. J. Plant Sci. (1988) 68:509-511). Stellar was 20% lower yielding than commercial cultivars at the time of its release.

Canola-quality oilseed Brassica varieties with reduced levels of saturated fatty acids in the seed oil could be used to produce food products which promote cardiovascular health. Canola lines which are individually low in palmitic and stearic acid content or low in combination will reduce the levels of saturated fatty acids. Similarly, Brassica varieties with increased monounsaturate levels in the seed oil, and products derived from such oil, would improve lipid nutrition. Canola lines which are low in linoleic acid tend to have high oleic acid content, and can be used in the development of varieties having even higher oleic acid content.

Increased palmitic acid content provides a functional improvement in food applications. Oils high in palmitic acid content are particularly useful in the

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formulation of margarines. Thus, there is a need for manufacturing purposes for oils high in palmitic acid content.

Decreased  $\alpha$ -linolenic acid content provides a functional improvement in food applications. Oils which are low in linolenic acid have increased stability. The rate of oxidation of lipid fatty acids increases with higher levels of linolenic acid leading to off-flavors and off-odors in foods. There is a need in the food industry for oils low in alpha linolenic acid.

Delta-12 fatty acid desaturase (also known as oleic desaturase) is involved in the enzymatic conversion of oleic acid to linoleic acid. Delta-15 fatty acid desaturase (also known as linoleic acid desaturase) is involved in the enzymatic conversion of linoleic acid to  $\alpha$ -linolenic acid. A microsomal delta-12 desaturase has been cloned and characterized using T-DNA tagging. Okuley, et al., Plant Cell 6:147-158 (1994). The nucleotide sequences of higher plant genes encoding microsomal delta-12 fatty acid desaturase are described in Lightner et al., WO94/11516. Sequences of higher plant genes encoding microsomal and plastid delta-15 fatty acid desaturases are disclosed in Yadav, N., et al., Plant Physiol., 103:467-476 (1993), WO 93/11245 and Arondel, V. et al., Science, 258:1353-1355 (1992). However, there are no teachings that disclose mutations in delta-12 or delta-15 fatty acid desaturase coding sequences from plants. Furthermore, no methods have been described for developing plant lines that contain delta-12 or delta-15 fatty acid desaturase gene sequence mutations effective for altering the fatty acid composition of seeds.

Summary of the Invention

The present invention comprises canola seeds, plant lines producing seeds, and plants producing seed, said seeds having a maximum content of FDA saturates of about 5% and a maximum erucic acid content of about 2% based upon total extractable oil and belonging to a line in which said saturates content has been stabilized for both the generation to which the seed belongs and its parent generation. Progeny of said seeds and canola oil having a maximum erucic acid content of about 2%, based upon total extractable oil, are additional aspects of this invention. Preferred are seeds, plant lines producing seeds, and plants producing seeds, said seeds having an FDA saturates content of from about 4.2% to about 5.0% based upon total extractable oil.

The present invention further comprises *Brassica* seeds, plant lines producing seeds, and plants producing seeds, said seeds having a minimum oleic acid content of about 71% based upon total extractable oil and belonging to a line in which said oleic acid content has been stabilized for both the generation to which the seed belongs and its parent generation. A further aspect of this invention is such high oleic acid seeds additionally having a maximum erucic acid content of about 2% based upon total extractable oil. Progeny of said seeds; and *Brassica* oil having 1) a minimum oleic acid content of about 71% or 2) a minimum oleic acid content of about 71% and a maximum erucic content of about 2% are also included in this invention. Preferred are seeds, plant lines producing seeds, and plants producing seeds, said seeds having an oleic acid content of from about 71.2% to about 78.3% based upon total extractable oil.

The present invention further comprises canola seeds, plant lines producing seeds, and plants producing seeds, said seeds having a maximum linoleic acid content

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of about 14% and a maximum erucic acid content of about 2% based upon total extractable oil and belonging to a line in which said acid content is stabilized for both the generation to which the seed belongs and its parent  
5 generation. Progeny of said seeds and canola oil having a maximum linoleic acid content of about 14% and a maximum erucic acid content of about 2%, are additional aspects of this invention. Preferred are seeds, plant lines producing seeds, and plants producing seeds, said  
10 seeds having a linoleic acid content of from about 8.4% to about 9.4% based upon total extractable oil.

The present invention further comprises *Brassica* seeds, plant lines producing seeds, and plants producing seeds, said seeds having a maximum palmitic acid content  
15 of about 3.5% and a maximum erucic acid content of about 2% based on total extractable oil and belonging to a line in which said acid content is stabilized for both the generation to which the seed belongs and its parent generation. Progeny of said seeds and canola having a  
20 maximum palmitic acid content of about 3.5% and a maximum erucic acid content of about 2%, are additional aspects of this invention. Preferred are seeds, plant lines producing seeds, and plants producing seeds, said seeds having a palmitic acid content of from about 2.7% to  
25 about 3.1% based upon total extractable oil.

The present invention further comprises *Brassica* seeds, plant lines producing seeds, and plants producing seeds, said seeds having a minimum palmitic acid content of about 9.0% based upon total extractable oil and  
30 belonging to a line in which said acid content is stabilized for both the generation to which the seed belongs and its parent generation. A further aspect of this invention is such high palmitic acid seeds additionally having a maximum erucic acid content of  
35 about 2% based upon total extractable oil. Progeny of



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said seeds; and Brassica oil having 1) a minimum palmitic acid content of about 9.0%, or 2) a minimum palmitic acid content of about 9.0% and a maximum erucic acid content of about 2% are also included in this invention.

- 5 Preferred are seeds, plant lines producing seeds, and plants producing seeds, said seeds having a palmitic acid content of from about 9.1% to about 11.7% based upon total extractable oil.

- The present invention further comprises Brassica  
10 seeds, plant lines producing seeds, and plants producing seeds, said seeds having a maximum stearic acid content of about 1.1% based upon total extractable oil and belonging to a line in which said acid content is stabilized for both the generation to which the seed  
15 belongs and its parent generation. Progeny of said seeds have a canola oil having a maximum stearic acid content of about 1.1% and maximum erucic acid content of about 2%. Preferred are seeds, plant lines producing seeds, and plants producing seeds having a palmitic acid content  
20 of from about 0.8% to about 1.1% based on total extractable oil.

- The present invention further comprises Brassica seeds, plant lines producing seeds, and plants producing seeds, said seeds having a sum of linoleic acid content  
25 and linolenic acid content of a maximum of about 14% based upon total extractable oil and belonging to a line in which said acid content is stabilized for both the generation to which the seed belongs and its parent generation. Progeny of said seeds have a canola oil  
30 having a sum of linoleic acid content and linolenic acid content of a maximum of about 14% and a maximum erucic acid content of about 2%. Preferred are seeds, plant lines producing seeds, and plants producing seeds having a sum of linoleic acid content and linolenic acid content

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of from about 11.8% to about 12.5% based on total extractable oil.

The invention further comprises *Brassicaceae* or *Helianthus* seeds, plants and plant lines having at least one mutation that controls the levels of unsaturated fatty acids in plants. One embodiment of the invention is an isolated nucleic acid fragment comprising a nucleotide sequence encoding a mutant delta-12 fatty acid desaturase conferring altered fatty composition in seeds when the fragment is present in a plant. A preferred sequence comprises a mutant sequence as shown in SEQ ID NO:3. Another embodiment of the invention is an isolated nucleic acid fragment comprising a nucleotide sequence encoding a mutant delta-15 fatty acid desaturase. A plant in this embodiment may be soybean, oilseed *Brassica* species, sunflower, castor bean or corn. The mutant sequence may be derived from, for example, a *Brassica napus*, *Brassica rapa*, *Brassica juncea* or *Helianthus* delta-12 or delta-15 gene.

Another embodiment of the invention involves a method of producing a *Brassicaceae* or *Helianthus* plant line comprising the steps of: (a) inducing mutagenesis in cells of a starting variety of a *Brassicaceae* or *Helianthus* species; (b) obtaining progeny plants from the mutagenized cells; (c) identifying progeny plants that contain a mutation in a delta-12 or delta-15 fatty acid desaturase gene; and (d) producing a plant line by selfing.

Yet another embodiment of the invention involves a method of producing plant lines containing altered levels of unsaturated fatty acids comprising: (a) crossing a first plant with a second plant having a mutant delta-12 or delta-15 fatty acid desaturase; (b) obtaining seeds from the cross of step (a); (c) growing fertile plants from such seeds; (d) obtaining progeny seed the plants of

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step (c); and (e) identifying those seeds among the progeny that have altered fatty acid composition. Suitable plants are soybean, rapeseed, sunflower, safflower, castor bean and corn. Preferred plants are rapeseed and sunflower.

The invention is also embodied in vegetable oil obtained from plants disclosed herein, which vegetable oil has an altered fatty acid composition.

#### Brief Description of the Figures

Figure 1 is a histogram showing the frequency distribution of seed oil oleic acid ( $C_{18:1}$ ) content in a segregating population of a Q508 X Westar cross. The bar labeled WSGA 1A represents the  $C_{18:1}$  content of the Westar parent. The bar labeled Q508 represents the  $C_{18:1}$  content of the Q508 parent.

#### Description of the Preferred Embodiments

The U.S. Food and Drug Administration defines saturated fatty acids as the sum of lauric ( $C_{12:0}$ ), myristic ( $C_{14:0}$ ), palmitic ( $C_{16:0}$ ) and stearic ( $C_{18:0}$ ) acids. The term "FDA saturates" as used herein means this above-defined sum. Unless total saturate content is specified, the saturated fatty acid values expressed here include only "FDA saturates."

All percent fatty acids herein are percent by weight of the oil of which the fatty acid is a component.

As used herein, a "line" is a group of plants that display little or no genetic variation between individuals for at least one trait. Such lines may be created by several generations of self-pollination and selection, or vegetative propagation from a single parent using tissue or cell culture techniques. As used herein, the term "variety" refers to a line which is used for commercial production.

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The term "mutagenesis" refers to the use of a mutagenic agent to induce random genetic mutations within a population of individuals. The treated population, or a subsequent generation of that population, is then  
5 screened for usable trait(s) that result from the mutations. A "population" is any group of individuals that share a common gene pool. As used herein "M<sub>1</sub>" is untreated seed. As used herein, "M<sub>1</sub>" is the seed (and resulting plants) exposed to a mutagenic agent, while "M<sub>2</sub>"  
10 is the progeny (seeds and plants) of self-pollinated M<sub>1</sub> plants, "M<sub>3</sub>" is the progeny of self-pollinated M<sub>2</sub> plants, and "M<sub>4</sub>" is the progeny of self-pollinated M<sub>3</sub> plants. "M<sub>5</sub>" is the progeny of self-pollinated M<sub>4</sub> plants. "M<sub>6</sub>", "M<sub>7</sub>", etc. are each the progeny of self-pollinated plants  
15 of the previous generation. The term "selfed" as used herein means self-pollinated.

"Stability" or "stable" as used herein means that with respect to a given fatty acid component, the component is maintained from generation to generation for  
20 at least two generations and preferably at least three generations at substantially the same level, e.g., preferably  $\pm 5\%$ . The method of invention is capable of creating lines with improved fatty acid compositions stable up to  $\pm 5\%$  from generation to generation. The  
25 above stability may be affected by temperature, location, stress and time of planting. Thus, comparison of fatty acid profiles should be made from seeds produced under similar growing conditions. Stability may be measured based on knowledge of prior generation.

30 Intensive breeding has produced *Brassica* plants whose seed oil contains less than 2% erucic acid. The same varieties have also been bred so that the defatted meal contains less than 30  $\mu\text{mol}$  glucosinolates/gram. "Canola" as used herein refers to plant variety seed or

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oil which contains less than 2% erucic acid ( $C_{22:1}$ ), and meal with less than 30  $\mu\text{mol}$  glucosinolates/gram.

Applicants have discovered plants with mutations in a delta-12 fatty acid desaturase gene. Such plants have useful alterations in the fatty acid compositions of the seed oil. Such mutations confer, for example, an elevated oleic acid content, a decreased, stabilized linoleic acid content, or both elevated oleic acid and decreased, stabilized linoleic acid content.

Applicants have further discovered plants with mutations in a delta-15 fatty acid desaturase gene. Such plants have useful alterations in the fatty acid composition of the seed oil, e.g., a decreased, stabilized level of  $\alpha$ -linolenic acid.

Applicants have further discovered isolated nucleic acid fragments comprising sequences that carry mutations within the coding sequence of delta-12 or delta-15 desaturases. The mutations confer desirable alterations in fatty acid levels in the seed oil of plants carrying such mutations. Delta-12 fatty acid desaturase is also known as omega-6 fatty acid desaturase and is sometimes referred to herein as 12-DES. Delta-15 fatty acid desaturase is also known as omega-3 fatty acid desaturase and is sometimes referred to herein as 15-DES.

A nucleic acid fragment of the invention contains a mutation in a microsomal delta-12 fatty acid desaturase coding sequence or in a microsomal delta-15 fatty acid desaturase coding sequence. Such a mutation renders the resulting desaturase gene product non-functional in plants, relative to the function of the gene product encoded by the wild-type sequence. The non-functionality of the 12-DES gene product can be inferred from the decreased level of reaction product (linoleic acid) and increased level of substrate (oleic acid) in plant tissues expressing the mutant sequence, compared to the

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corresponding levels in plant tissues expressing the wild-type sequence. The non-functionality of the 15-DES gene product can be inferred from the decreased level of reaction product ( $\alpha$ -linolenic acid) and the increased  
5 level of substrate (linoleic acid) in plant tissues expressing the mutant sequence, compared to the corresponding levels in plant tissues expressing the wild-type sequence.

A nucleic acid fragment of the invention may  
10 comprise a portion of the coding sequence, e.g., at least about 10 nucleotides, provided that the fragment contains at least one mutation in the coding sequence. The length of a desired fragment depends upon the purpose for which the fragment will be used, e.g., PCR primer, site-  
15 directed mutagenesis and the like. In one embodiment, a nucleic acid fragment of the invention comprises the full length coding sequence of a mutant delta-12 or mutant delta-15 fatty acid desaturase.

A mutation in a nucleic acid fragment of the  
20 invention may be in any portion of the coding sequence that renders the resulting gene product non-functional. Suitable types of mutations include, without limitation, insertions of nucleotides, deletions of nucleotides, or transitions and transversions in the wild-type coding  
25 sequence. Such mutations result in insertions of one or more amino acids, deletions of one or more amino acids, and non-conservative amino acid substitutions in the corresponding gene product. In some embodiments, the sequence of a nucleic acid fragment may comprise more  
30 than one mutation or more than one type of mutation.

Insertion or deletion of amino acids in a coding sequence may, for example, disrupt the conformation of essential alpha-helical or beta-pleated sheet regions of the resulting gene product. Amino acid insertions or  
35 deletions may also disrupt binding or catalytic sites

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important for gene product activity. It is known in the art that the insertion or deletion of a larger number of contiguous amino acids is more likely to render the gene product non-functional, compared to a smaller number of inserted or deleted amino acids.

Non-conservative amino acid substitutions may replace an amino acid of one class with an amino acid of a different class. Non-conservative substitutions may make a substantial change in the charge or hydrophobicity of the gene product. Non-conservative amino acid substitutions may also make a substantial change in the bulk of the residue side chain, e.g., substituting an alanyl residue for a isoleucyl residue.

Examples of non-conservative substitutions include the substitution of a basic amino acid for a non-polar amino acid, or a polar amino acid for an acidic amino acid. Because there are only 20 amino acids encoded in a gene, substitutions that result in a non-functional gene product may be determined by routine experimentation, incorporating amino acids of a different class in the region of the gene product targeted for mutation.

Preferred mutations are in a region of the nucleic acid having an amino acid sequence motif that is conserved among delta-12 fatty acid desaturases or delta-15 fatty acid desaturases, such as a His-Xaa-Xaa-Xaa-His motif (Tables 1-3). An example of a suitable region has a conserved HECGH motif that is found, for example, in nucleotides corresponding to amino acids 105 to 109 of the *Arabidopsis* and *Brassica* delta-12 desaturase sequences, in nucleotides corresponding to amino acids 101 to 105 of the soybean delta-12 desaturase sequence and in nucleotides corresponding to amino acids 111 to 115 of the maize delta-12 desaturase sequence. See e.g., WO 94/115116; Okuley et al., Plant Cell 6:147-158 (1994). The one letter amino acid designations used herein are

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described in Alberts, B. et al., Molecular Biology of the Cell, 3rd edition, Garland Publishing, New York, 1994. Amino acids flanking this motif are also highly conserved among delta-12 and delta-15 desaturases and are also suitable candidates for mutations in fragments of the invention. An illustrative embodiment of a mutation in a nucleic acid fragment of the invention is a Glu to Lys substitution in the HECGH motif of a *Brassica* microsomal delta-12 desaturase sequence, either the D form or the F form. This mutation results in the sequence HECGH being changed to HKCGH as seen by comparing SEQ ID NO:2 (wild-type D form) to SEQ ID NO:4 (mutant D form).

A similar motif may be found at amino acids 101 to 105 of the *Arabidopsis* microsomal delta-15 fatty acid desaturase, as well as in the corresponding rape and soybean desaturases (Table 5). See, e.g., WO 93/11245; Arondel, V. et al., Science, 258:1153-1155 (1992); Yadav, N. et al., Plant Physiol., 103:467-476 (1993). Plastid delta-15 fatty acids have a similar motif (Table 5).

Among the types of mutations in an HECGH motif that render the resulting gene product non-functional are non-conservative substitutions. An illustrative example of a non-conservative substitution is substitution of a glycine residue for either the first or second histidine. Such a substitution replaces a polar residue (histidine) with a non-polar residue (glycine). Another type of mutation that renders the resulting gene product non-functional is an insertion mutation, e.g., insertion of a glycine between the cystine and glutamic acid residues in the HECGH motif.

Other regions having suitable conserved amino acid motifs include the HRRHH motif shown in Table 2, the HRTTH motif shown in Table 6 and the HVAHH motif shown in Table 3. See, e.g., WO 94/115116; Hitz, W. et al., Plant



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Physiol., 105:635-641 (1994); Okuley, J., et al., supra; and Yadav, N. et al., supra.

Another region suitable for a mutation in a delta-12 desaturase sequence contains the motif KYLNNP at 5 nucleotides corresponding to amino acids 171 to 175 of the *Brassica* desaturase sequence. An illustrative example of a mutation in this region is a Leu to His substitution, resulting in the amino acid sequence (Table 4) KYHNN (Compare wild-type SEQ ID NO:6 to mutant SEQ ID 10 NO:8).

**TABLE 1**

Alignment of Amino Acid Sequences from Microsomal  
Delta-12 Fatty Acid Desaturases

	<u>Species</u>	<u>Position</u>	<u>Amino Acid Sequence</u>
15	<i>Arabidopsis thaliana</i>	100-129	IWVIAHECGH HAFSDYQWLD DTVGLIFHSF
	<i>Glycine max</i>	96-125	VWVIAHECGH HAFSKYQWVD DVVGLTLHST
	<i>Zea mays</i>	106-135	VWVIAHECGH HAFSDYSLLD DVVGLVLHSS
	<i>Ricinus communis</i> <sup>a</sup>	1- 29	WVMAHDCGH HAFSDYQLLD DVVGLILHSC
	<i>Brassica napus D</i>	100-128	VWVIAHECGH HAFSDYQWLD DTVGLIFHS
20	<i>Brassica napus F</i>	100-128	VWVIAHECGH HAFSDYQWLD DTVGLIFHS

<sup>a</sup> from plasmid pRF2-1C

**TABLE 2**

Alignment of Amino Acid Sequences from Microsomal  
Delta-12 Fatty Acid Desaturases

	<u>Species</u>	<u>Position</u>	<u>Amino Acid Sequence</u>
25	<i>Arabidopsis thaliana</i>	130-158	LLVPYFSWKY SHRRHHSNTG SLERDEVFV
	<i>Glycine max</i>	126-154	LLVPYFSWKI SHRRHHSNTG SLDRDEVFV
	<i>Zea mays</i>	136-164	LMVPYFSWKY SHRRHHSNTG SLERDEVFV
	<i>Ricinus communis</i> <sup>a</sup>	30- 58	LLVPYFSWKH SHRRHHSNTG SLERDEVFV
30	<i>Brassica napus D</i>	130-158	LLVPYFSWKY SHRSHHSNTG SLERDEVFV
	<i>Brassica napus F</i>	130-158	LLVPYFSWKY SHRRHHSNTG SLERDEVFV

<sup>a</sup> from plasmid pRF2-1C

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**TABLE 3**

Alignment of Amino Acid Sequences from Microsomal  
Delta-12 Fatty Acid Desaturases

	<u>Species</u>	<u>Position</u>	<u>Amino Acid Sequence</u>
5	<i>Arabidopsis thaliana</i>	298-333	DRDYGILNKV FHNITDTHVA HHLFSTMPHY NAMEAT
	<i>Glycine max</i>	294-329	DRDYGILNKV FHNITDTHVA HHLFSTMPHY HAMEAT
	<i>Zea mays</i>	305-340	DRDYGILNRV FHNITDTHVA HHLFSTMPHY HAMEAT
	<i>Ricinus communis</i> *	198-224	DRDYGILNKV FHNITDTQVA HHLF TMP
	<i>Brassica napus D</i>	299-334	DRDYGILNKV FHNITDTHVA HHPFSTMPHY HAMEAT
10	<i>Brassica napus F</i>	299-334	DRDYGILNKV FHNITDTHVA HHLFSTMPHY HAMEAT

from plasmid pRF2-1C

**TABLE 4**

Alignment of Conserved Amino Acids from Microsomal  
Delta-12 Fatty Acid Desaturases

	<u>Species</u>	<u>Position</u>	<u>Amino Acid Sequence</u>
15	<i>Arabidopsis thaliana</i>	165-180	IKWYGKYLNN PLGRIM
	<i>Glycine max</i>	161-176	VAWFSLYLNN PLGRAV
	<i>Zea mays</i>	172-187	PWYTPYVYNN PVGRVV
	<i>Ricinus communis</i> *	65- 80	IRWYSKYLNN PPGRIM
	<i>Brassica napus D</i>	165-180	IKWYGKYLNN PLGRTV
20	<i>Brassica napus F</i>	165-180	IKWYGKYLNN PLGRTV

\* from plasmid pRF2-1C

**TABLE 5**

Alignment of Conserved Amino Acids from Plastid and Microsomal  
Delta-15 Fatty Acid Desaturases

	<u>Species</u>	<u>Position</u>	<u>Amino Acid Sequence</u>
25	<i>Arabidopsis thaliana</i> *	156-177	WALFVLGHD CGHGSFSNDP KLN
	<i>Brassica napus</i> *	114-135	WALFVLGHD CGHGSFSNDP RLN
	<i>Glycine max</i> *	164-185	WALFVLGHD CGHGSFSNNS KLN
	<i>Arabidopsis thaliana</i>	94-115	WAIFVLGHD CGHGSFSDIP LLN
	<i>Brassica napus</i>	87-109	WALFVLGHD CGHGSFSNDP RLN
30	<i>Glycine max</i>	93-114	WALFVLGHD CGHGSFSDSP PLN

\* Plastid sequences

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**TABLE 6**

Alignment of Conserved Amino Acids from Plastid and Microsomal  
Delta-15 Fatty Acid Desaturases

	<u>Species</u>	<u>Position</u>	<u>Amino Acid Sequence</u>
5	<i>A. thaliana</i> <sup>a</sup>	188-216	ILVPYHGWRI SHRTHHQNHG HVENDESWH
	<i>B. napus</i> <sup>a</sup>	146-174	ILVPYHGWRI SHRTHHQNHG HVENDESWH
	<i>Glycine max</i> <sup>a</sup>	196-224	ILVPYHGWRI SHRTHHQNHG HAENDESWH
	<i>A. thaliana</i>	126-154	ILVPYHGWRI SHRTHHQNHG HVENDESWV
	<i>Brassica napus</i>	117-145	ILVPYHGWRI SHRTHHQNHG HVENDESWV
10	<i>Glycine max</i>	125-153	ILVPYHGWRI SHRTHHQNHG HIEKDESWV

<sup>a</sup> Plastid sequences

The conservation of amino acid motifs and their relative positions indicates that regions of a delta-12 or delta-15 fatty acid desaturase that can be mutated in one species to generate a non-functional desaturase can be mutated in the corresponding region from other species to generate a non-functional 12-DES or 15-DES gene product in that species.

Mutations in any of the regions of Tables 1-6 are specifically included within the scope of the invention, provided that such mutation (or mutations) renders the resulting desaturase gene product non-functional, as discussed hereinabove.

A nucleic acid fragment containing a mutant sequence can be generated by techniques known to the skilled artisan. Such techniques include, without limitation, site-directed mutagenesis of wild-type sequences and direct synthesis using automated DNA synthesizers.

A nucleic acid fragment containing a mutant sequence can also be generated by mutagenesis of plant seeds or regenerable plant tissue by, e.g., ethyl methane sulfonate, X-rays or other mutagens. With mutagenesis, mutant plants having the desired fatty acid phenotype in seeds are identified by known techniques and a nucleic acid fragment containing the desired mutation is isolated from genomic DNA or RNA of the mutant line. The site of

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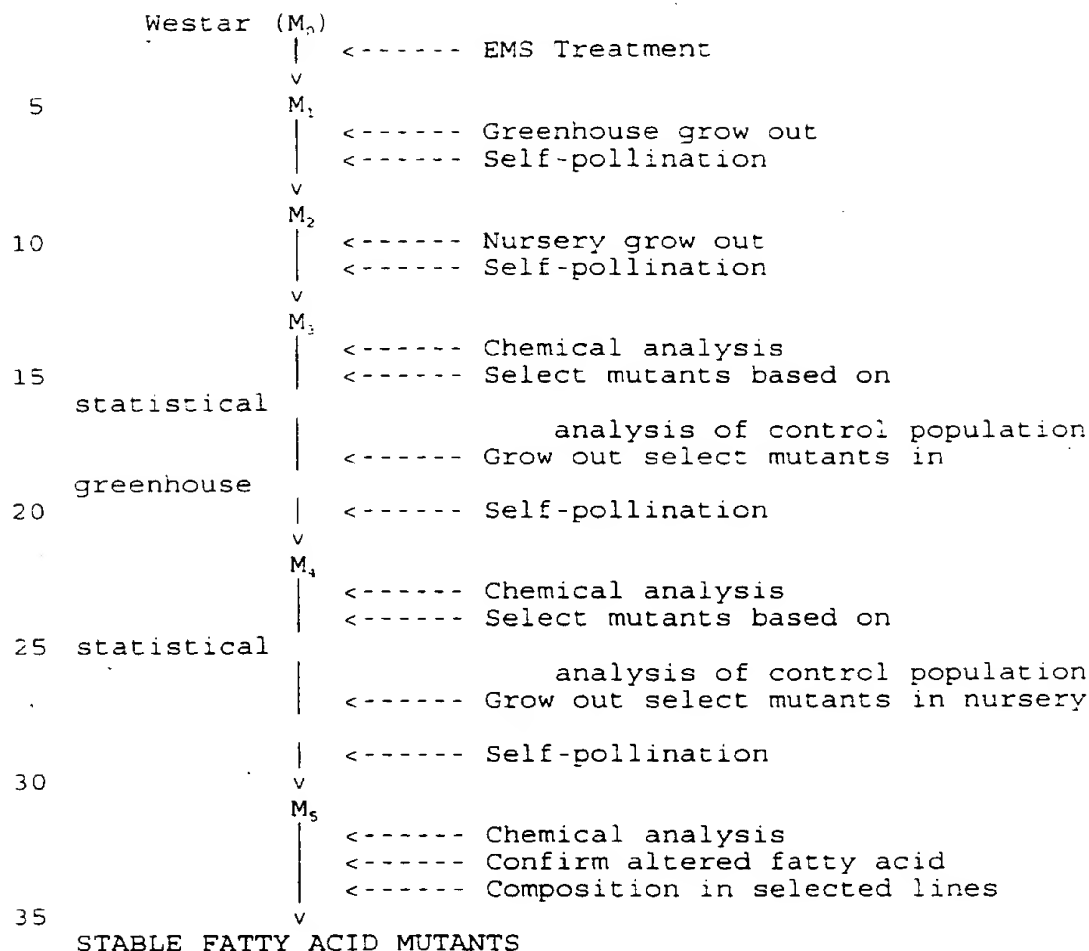
the specific mutation is then determined by sequencing the coding region of the 12-DES or 15-DES gene.

Alternatively, labeled nucleic acid probes that are specific for desired mutational events can be used to rapidly screen a mutagenized population.

Seeds of Westar, a Canadian (*Brassica napus*) spring canola variety, were subjected to chemical mutagenesis. Mutagenized seeds were planted in the greenhouse and the plants were self-pollinated. The progeny plants were individually analyzed for fatty acid composition, and regrown either in the greenhouse or in the field. After four successive generations of self-pollinations, followed by chemical analysis of the seed oil at each cycle, several lines were shown to carry stably inherited mutations in specific fatty acid components, including reduced palmitic acid ( $C_{16:0}$ ), increased palmitic acid, reduced stearic acid ( $C_{18:0}$ ), increased oleic acid ( $C_{18:1}$ ), reduced linoleic acid ( $C_{18:2}$ ) and reduced linolenic acid ( $C_{18:3}$ ), in the seed oil.

The general experimental scheme for developing lines with stable fatty acid mutations is shown in Scheme I hereinafter.

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SCHEME I

Westar seeds ( $M_0$ ) were mutagenized with ethylmethanesulfonate (EMS). Westar is a registered Canadian spring variety with canola quality. The fatty acid composition of field-grown Westar, 3.9%  $C_{16:0}$ , 1.9%  $C_{18:0}$ , 67.5%  $C_{18:1}$ , 17.6%  $C_{18:2}$ , 7.4%  $C_{18:3}$ , <2%  $C_{20:1}$  +  $C_{22:1}$ , has remained stable under commercial production, with  $\pm 10\%$  deviation, since 1982. The disclosed method may be applied to all oilseed Brassica species, and to both Spring and Winter maturing types within each species. Physical mutagens, including but not limited to X-rays,

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UV rays, and other physical treatments which cause chromosome damage, and other chemical mutagens, including but not limited to ethidium bromide, nitrosoguanidine, diepoxybutane etc. may also be used to induce mutations.

- 5 The mutagenesis treatment may also be applied to other stages of plant development, including but not limited to cell cultures, embryos, microspores and shoot apices. The  $M_1$  seeds were planted in the greenhouse and  $M_1$  plants were individually self-pollinated.

- 10  $M_2$  seed was harvested from the greenhouse and planted in the field in a plant-to-row design. Each plot contained six rows, and five  $M_2$  lines were planted in each plot. Every other plot contained a row of non-mutagenized Westar as a control. Based on gas  
15 chromatographic analysis of  $M_2$  seed, those lines which had altered fatty acid composition were self-pollinated and individually harvested.

- $M_3$  seeds were evaluated for mutations on the basis of a Z-distribution. An extremely stringent 1 in 10,000  
20 rejection rate was employed to establish statistical thresholds to distinguish mutation events from existing variation. Mean and standard deviation values were determined from the non-mutagenized Westar control population in the field. The upper and lower statistical  
25 thresholds for each fatty acid were determined from the mean value of the population  $\pm$  the standard deviation, multiplied by the Z-distribution. Based on a population size of 10,000, the confidence interval is 99.99%.

- Seeds ( $M_3$ ) from those  $M_2$  lines which exceeded  
30 either the upper or lower statistical thresholds were replanted in the greenhouse and self-pollinated. This planting also included Westar controls. The  $M_4$  seed was re-analyzed using new statistical thresholds established with a new control population. Those  $M_4$  lines which  
35 exceeded the new statistical thresholds for selected

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fatty acid compositions were advanced to the nursery. Following self-pollination,  $M_3$  seed from the field were re-analyzed once again for fatty acid composition. Those lines which remained stable for the selected fatty acids  
5 were considered stable mutations.

"Stable mutations" as used herein are defined as  $M_3$  or more advanced lines which maintain a selected altered fatty acid profile for a minimum of three generations, including a minimum of two generations under  
10 field conditions, and exceeding established statistical thresholds for a minimum of two generations, as determined by gas chromatographic analysis of a minimum of 10 randomly selected seeds bulked together. Alternatively, stability may be measured in the same way  
15 by comparing to subsequent generations. In subsequent generations, stability is defined as having similar fatty acid profiles in the seed as that of the prior or subsequent generation when grown under substantially similar conditions.

20 The amount of variability for fatty acid content in a seed population is quite significant when single seeds are analyzed. Randomly selected single seeds and a ten seed bulk sample of a commercial variety were compared. Significant variation among the single seeds  
25 was detected (Table A). The half-seed technique (Downey, R.K. and B.L. Harvey, Can. J. Plant Sci., 43:271 [1963]) in which one cotyledon of the germinating seed is analyzed for fatty acid composition and the remaining embryo grown into a plant has been very useful to plant  
30 breeding work to select individuals in a population for further generation analysis. The large variation seen in the single seed analysis (Table A) is reflected in the half-seed technique.

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TABLE A

Single Seed Analysis for Fatty Acid Composition<sup>1</sup>

	SAMPLE	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1
5	Bulk	3.2	0.4	1.8	20.7	13.7	9.8	0.8	11.2	0.4	32.2
	1	2.8	0.2	1.1	14.6	14.6	11.1	0.8	9.8	0.7	38.3
	2	3.3	0.2	1.3	13.1	14.4	11.7	0.9	10.5	0.7	37.0
	3	3.0	--	1.2	12.7	15.3	10.6	0.8	7.3	0.7	43.2
	4	2.8	0.2	1.1	16.7	13.2	9.1	0.8	11.2	0.4	38.9
10	5	3.0	--	1.8	15.2	13.3	8.4	1.3	8.7	0.9	42.3
	6	3.1	--	1.3	14.4	14.6	10.3	1.0	10.9	0.8	39.3
	7	2.6	--	1.2	15.7	13.8	9.9	0.9	12.2	0.5	37.0
	8	3.1	--	1.1	16.2	13.4	10.6	0.6	9.2	0.8	41.4
	9	2.7	0.1	1.0	13.5	11.2	11.3	0.8	5.2	0.7	46.9
15	10	3.4	0.2	1.4	13.9	17.5	10.8	1.1	10.0	0.9	36.2
	11	2.8	0.2	1.2	12.7	12.9	10.3	1.0	7.9	0.9	43.3
	12	2.3	0.1	1.6	20.7	14.8	6.5	1.1	12.5	0.8	34.5
	13	2.6	0.2	1.3	21.0	11.4	7.6	1.0	11.6	0.6	36.7
	14	2.6	0.1	1.2	14.7	13.2	9.4	0.9	10.1	0.8	40.8
20	15	2.9	0.2	1.4	16.6	15.1	11.2	0.7	9.1	0.3	36.1
	16	3.0	0.2	1.1	12.4	13.7	10.4	0.9	8.7	0.8	42.7
	17	2.9	0.1	1.1	21.1	12.3	7.1	0.8	12.4	0.5	36.8
	18	3.1	0.1	1.2	13.7	13.1	10.4	1.0	8.8	0.7	41.6
	19	2.7	0.1	1.0	11.1	13.4	11.7	0.8	7.9	0.8	43.5
25	20	2.3	0.2	0.2	18.2	13.9	8.2	0.9	10.3	0.8	38.2
	Average	2.8	0.2	1.2	15.4	13.8	9.8	0.9	9.8	0.7	39.7
	Minimum	2.3	0.1	0.2	11.1	11.2	6.5	0.6	6.2	0.3	34.5
	Maximum	3.4	0.2	1.8	21.1	17.5	11.7	1.3	12.5	0.9	46.9
	Range	1.1	0.1	1.6	9.9	6.3	5.3	0.7	6.4	0.6	12.4

Values expressed as percent of total oil

30 Plant breeders using the half-seed technique have found it unreliable in selecting stable genetically controlled fatty acid mutations (Stefanson, B.R., In; High and Low Erucic Acid Rapeseed Oils, Ed. N.T. Kenthies, Academic Press, Inc., Canada (1983) pp. 145-  
 35 159). Although valuable in selecting individuals from a population, the selected traits are not always transmitted to subsequent generations (Rakow, G. and McGregor, D.I., J. Amer. Oil Chem. Soc. (1973) 50:400-403. To determine the genetic stability of the selected  
 40 plants several self-pollinated generations are required (Robelen, G. In: Biotechnology for the Oils and Fats Industry, Ed. C. Ratledge, P. Dawson and J. Rattray,



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American Oil Chemists Society (1984) pp. 97-105) with chemical analysis of a bulk seed sample.

Mutation breeding has traditionally produced plants carrying, in addition to the trait of interest, multiple, deleterious traits, e.g., reduced plant vigor and reduced fertility. Such traits may indirectly affect fatty acid composition, producing an unstable mutation; and/or reduce yield, thereby reducing the commercial utility of the invention. To eliminate the occurrence of deleterious mutations and reduce the load of mutations carried by the plant a low mutagen dose was used in the seed treatments to create an LD30 population. This allowed for the rapid selection of single gene mutations for fatty acid traits in agronomic backgrounds which produce acceptable yields.

Other than changes in the fatty acid composition of the seed oil, the mutant lines described here have normal plant phenotype when grown under field conditions, and are commercially useful. "Commercial utility" is defined as having a yield, as measured by total pounds of seed or oil produced per acre, within 15% of the average yield of the starting ( $M_0$ ) canola variety grown in the same region. To be commercially useful, plant vigor and high fertility are such that the crop can be produced in this yield by farmers using conventional farming equipment, and the oil with altered fatty acid composition can be extracted using conventional crushing and extraction equipment.

The seeds of several different fatty acid lines have been deposited with the American Type Culture Collection and have the following accession numbers.

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	<u>Line</u>	<u>Accession No.</u>	<u>Deposit Date</u>
	A129.5	40811	May 25, 1990
	A133.1	40812	May 25, 1990
	A144.1	40813	May 25, 1990
5	A200.7	40816	May 31, 1990
	M3032.1	75021	June 7, 1991
	M3094.4	75023	June 7, 1991
	M3052.6	75024	June 7, 1991
	M3007.4	75022	June 7, 1991
10	M3062.8	75025	June 7, 1991
	M3028.10	75026	June 7, 1991
	IMC130	75446	April 16, 1993

In some plant species or varieties more than one form of endogenous microsomal delta-12 desaturase may be found. In amphidiploids, each form may be derived from one of the parent genomes making up the species under consideration. Plants with mutations in both forms have a fatty acid profile that differs from plants with a mutation in only one form. An example of such a plant is *Brassica napus* line Q508, a doubly-mutagenized line containing a mutant D-form of delta-12 desaturase (SEQ ID NO:1) and a mutant F-form of delta-12 desaturase (SEQ ID NO:5).

Preferred host or recipient organisms for introduction of a nucleic acid fragment of the invention are the oil-producing species, such as soybean (*Glycine max*), rapeseed (e.g., *Brassica napus*, *B. rapa* and *B. juncea*), sunflower (*Helianthus annuus*), castor bean (*Ricinus communis*), corn (*Zea mays*), and safflower (*Carthamus tinctorius*).

Plants according to the invention preferably contain an altered fatty acid profile. For example, oil obtained from seeds of such plants may have from about 69 to about 90% oleic acid, based on the total fatty acid composition of the seed. Such oil preferably has from about 74 to about 90% oleic acid, more preferably from about 80 to about 90% oleic acid. In some embodiments, oil obtained from seeds produced by plants of the

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invention may have from about 2.0% to about 5.0% saturated fatty acids, based on total fatty acid composition of the seeds. In some embodiments, oil obtained from seeds of the invention may have from about 5 1.0% to about 14.0% linoleic acid, or from about 0.5% to about 10.0%  $\alpha$ -linolenic acid.

In one embodiment of the claimed invention, a plant contains both a 12-DES mutation and a 15-DES mutation. Such plants can have a fatty acid composition 10 comprising very high oleic acid and very low alpha-linolenic acid levels. Mutations in 12-DES and 15-DES may be combined in a plant by making a genetic cross between 12-DES and 15-DES single mutant lines. A plant having a mutation in delta-12 fatty acid desaturase is 15 crossed or mated with a second plant having a mutation in delta-15 fatty acid desaturase. Seeds produced from the cross are planted and the resulting plants are selfed in order to obtain progeny seeds. These progeny seeds are then screened in order to identify those seeds carrying 20 both mutant genes.

Alternatively, a line possessing either a 12-DES or a 15-DES mutation can be subjected to mutagenesis to generate a plant or plant line having mutations in both 12-DES and 15-DES. For example, the IMC 129 line has a 25 mutation in the coding region (Glu<sub>106</sub> to Lys<sub>106</sub>) of the D form of the microsomal delta-12 desaturase structural gene. Cells (e.g., seeds) of this line can be mutagenized to induce a mutation in a 15-DES gene, resulting in a plant or plant line carrying a mutation in 30 a delta-12 fatty acid desaturase gene and a mutation in a delta-15 fatty acid desaturase gene.

Progeny includes descendants of a particular plant or plant line, e.g., seeds developed on an instant plant. Progeny of an instant plant include seeds formed on F<sub>1</sub>.

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$F_2$ ,  $F_3$ , and subsequent generation plants, or seeds formed on  $BC_1$ ,  $BC_2$ ,  $BC_3$  and subsequent generation plants.

Those seeds having an altered fatty acid composition may be identified by techniques known to the skilled artisan, e.g., gas-liquid chromatography (GLC) analysis of a bulked seed sample or of a single half-seed. Half-seed analysis is well known in the art to be useful because the viability of the embryo is maintained and thus those seeds having a desired fatty acid profile may be planted to form the next generation. However, half-seed analysis is also known to be an inaccurate representation of genotype of the seed being analyzed. Bulk seed analysis typically yields a more accurate representation of the fatty acid profile of a given genotype.

The nucleic acid fragments of the invention can be used as markers in plant genetic mapping and plant breeding programs. Such markers may include restriction fragment length polymorphism (RFLP), random amplification polymorphism detection (RAPD), polymerase chain reaction (PCR) or self-sustained sequence replication (SSR) markers, for example. Marker-assisted breeding techniques may be used to identify and follow a desired fatty acid composition during the breeding process. Marker-assisted breeding techniques may be used in addition to, or as an alternative to, other sorts of identification techniques. An example of marker-assisted breeding is the use of PCR primers that specifically amplify a sequence containing a desired mutation in 12-DES or 15-DES.

Methods according to the invention are useful in that the resulting plants and plant lines have desirable seed fatty acid compositions as well as superior agronomic properties compared to known lines having altered seed fatty acid composition. Superior agronomic

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characteristics include, for example, increased seed germination percentage, increased seedling vigor, increased resistance to seedling fungal diseases (damping off, root rot and the like), increased yield, and  
5 improved standability.

While the invention is susceptible to various modifications and alternative forms, certain specific embodiments thereof are described in the general methods and examples set forth below. For example the invention  
10 may be applied to all *Brassica* species, including *B. rapa*, *B. juncea*, and *B. hirta*, to produce substantially similar results. It should be understood, however, that these examples are not intended to limit the invention to the particular forms disclosed but, instead the invention  
15 is to cover all modifications, equivalents and alternatives falling within the scope of the invention. This includes the use of somaclonal variation; physical or chemical mutagenesis of plant parts; anther, microspore or ovary culture followed by chromosome  
20 doubling; or self- or cross-pollination to transmit the fatty acid trait, alone or in combination with other traits, to develop new *Brassica* lines.

#### EXAMPLE 1

##### Selection of Low FDA Saturates

25 Prior to mutagenesis, 30,000 seeds of *B. napus* cv. Westar seeds were preimbibed in 300-seed lots for two hours on wet filter paper to soften the seed coat. The preimbibed seeds were placed in 80 mM ethylmethanesulfonate (EMS) for four hours. Following  
30 mutagenesis, the seeds were rinsed three times in distilled water. The seeds were sown in 48-well flats containing Pro-Mix. Sixty-eight percent of the mutagenized seed germinated. The plants were maintained at 25°C/15°C, 14/10 hr day/night conditions in the

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greenhouse. At flowering, each plant was individually self-pollinated.

$M_2$  seed from individual plants were individually catalogued and stored, approximately 15,000  $M_2$  lines was  
5 planted in a summer nursery in Carman, Manitoba. The seed from each selfed plant were planted in 3-meter rows with 6-inch row spacing. Westar was planted as the check variety. Selected lines in the field were selfed by bagging the main raceme of each plant. At maturity, the  
10 selfed plants were individually harvested and seeds were catalogued and stored to ensure that the source of the seed was known.

Self-pollinated  $M_1$  seed and Westar controls were analyzed in 10-seed bulk samples for fatty acid  
15 composition via gas chromatography. Statistical thresholds for each fatty acid component were established using a Z-distribution with a stringency level of 1 in 10,000. The selected  $M_3$  seeds were planted in the greenhouse along with Westar controls. The seed was sown  
20 in 4-inch pots containing Pro-Mix soil and the plants were maintained at 25°C/15°C, 14/10 hr day/night cycle in the greenhouse. At flowering, the terminal raceme was self-pollinated by bagging. At maturity, selfed  $M_4$  seed was individually harvested from each plant, labelled, and  
25 stored to ensure that the source of the seed was known.

The  $M_4$  seed was analyzed in 10-seed bulk samples. Statistical thresholds for each fatty acid component were established from 259 control samples using a Z-distribution of 1 in 800. Selected  $M_4$  lines were planted  
30 in a field trial in Carman, Manitoba in 3-meter rows with 6-inch spacing. Ten  $M_4$  plants in each row were bagged for self-pollination. At maturity, the selfed plants were individually harvested and the open pollinated plants in the row were bulk harvested. The  $M_5$  seed from single

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plant selections was analyzed in 10-seed bulk samples and the bulk row harvest in 50-seed bulk samples.

- Selected  $M_2$  lines were planted in the greenhouse along with Westar controls. The seed was grown as previously described. At flowering the terminal raceme was self-pollinated by bagging. At maturity, selfed  $M_2$  seed was individually harvested from each plant and analyzed in 10-seed bulk samples for fatty acid composition.
- Selected  $M_2$  lines were entered into field trials in Eastern Idaho. The four trial locations were selected for the wide variability in growing conditions. The locations included Burley, Tetonia, Lamont and Shelley (Table I). The lines were planted in four 3-meter rows with an 8-inch spacing, each plot was replicated four times. The planting design was determined using a Randomized Complete Block Design. The commercial cultivar Westar was used as a check cultivar. At maturity the plots were harvested to determine yield. Yield of the entries in the trial was determined by taking the statistical average of the four replications. The Least Significant Difference Test was used to rank the entries in the randomized complete block design.

**TABLE I**

**Trial Locations for Selected Fatty Acid Mutants**

<u>LOCATION</u>	<u>SITE CHARACTERIZATIONS</u>
BURLEY	Irrigated. Long season. High temperatures during flowering.
TETONIA	Dryland. Short season. Cool temperatures.
LAMONT	Dryland. Short season. Cool temperatures.
SHELLEY	Irrigated. Medium season. High temperatures during flowering.

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To determine the fatty acid profile of entries, plants in each plot were bagged for self-pollination. The  $M_1$  seed from single plants was analyzed for fatty acids in ten-seed bulk samples.

5 To determine the genetic relationships of the selected fatty acid mutants crosses were made. Flowers of  $M_1$  or later generation mutations were used in crossing.  $F_1$  seed was harvested and analyzed for fatty acid composition to determine the mode of gene action. The  $F_1$  progeny were planted in the greenhouse. The resulting plants were self-pollinated, the  $F_2$  seed harvested and analyzed for fatty acid composition for allelism studies. The  $F_2$  seed and parent line seed was planted in the greenhouse, individual plants were self-pollinated. The  $F_3$  seed of individual plants was tested for fatty acid composition using 10-seed bulk samples as described previously.

In the analysis of some genetic relationships dihaploid populations were made from the microspores of the  $F_1$  hybrids. Self-pollinated seed from dihaploid plants were analyzed for fatty acid analysis using methods described previously.

For chemical analysis, 10-seed bulk samples were hand ground with a glass rod in a 15-mL polypropylene tube and extracted in 1.2 mL 0.25 N KOH in 1:1 ether/methanol. The sample was vortexed for 30 sec. and heated for 60 sec. in a 60°C water bath. Four mL of saturated NaCl and 2.4 mL of iso-octane were added, and the mixture was vortexed again. After phase separation, 600  $\mu$ L of the upper organic phase were pipetted into individual vials and stored under nitrogen at -5°C. One  $\mu$ L samples were injected into a Supelco SP-2330 fused silica capillary column (0.25 mm ID, 30 M length, 0.20  $\mu$ m df).



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The gas chromatograph was set at 180°C for 5.5 minutes, then programmed for a 2°C/minute increase to 212°C, and held at this temperature for 1.5 minutes. Total run time was 23 minutes. Chromatography settings were: Column head pressure - 15 psi, Column flow (He) - 0.7 mL/min., Auxiliary and Column flow - 33 mL/min., Hydrogen flow - 33 mL/min., Air flow - 400 mL/min., Injector temperature - 250°C, Detector temperature - 300°C, Split vent - 1/15.

Table II describes the upper and lower statistical thresholds for each fatty acid of interest.

**TABLE II**

Statistical Thresholds for Specific Fatty Acids  
Derived from Control Westar Plantings

Genotype	Percent Fatty Acids					
	C <sub>16:0</sub>	C <sub>18:0</sub>	C <sub>18:1</sub>	C <sub>18:2</sub>	C <sub>19:0</sub>	Sats*
M <sub>3</sub> Generation (1 in 10,000 rejection rate)						
Lower	3.3	1.4	--	13.2	5.3	6.0
Upper	4.3	2.5	71.0	21.6	9.9	8.3
M <sub>4</sub> Generation (1 in 800 rejection rate)						
Lower	3.6	0.8	--	12.2	3.2	5.3
Upper	6.3	3.1	76.0	32.4	9.9	11.2
M <sub>5</sub> Generation (1 in 755 rejection rate)						
Lower	2.7	0.9	--	9.6	2.6	4.5
Upper	5.7	2.7	80.3	26.7	9.6	10.0

\*Sats=Total Saturate Content

At the M<sub>3</sub> generation, twelve lines exceeded the lower statistical threshold for palmitic acid ( $\leq 3.3\%$ ).

Line W13097.4 had 3.1% palmitic acid and an FDA saturate content of 4.5%. After a cycle in the greenhouse, M<sub>4</sub> seed

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from line W13097.4 (designated line A144) was analyzed. Line W13097.4.1(A144.1) had 3.1%  $C_{16:0}$ , exceeding the lower statistical threshold of 3.6%. The FDA saturate content for A144.1 was 4.5%. The fatty acid compositions for the 5  $M_3$ ,  $M_4$  and  $M_5$  generations of this family are summarized in Table III.

**TABLE III**

Fatty Acid Composition of a Low Palmitic Acid/Low FDA Saturate Canola Line Produced by Seed Mutagenesis

Genotype <sup>a</sup>	Percent Fatty Acids						
	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	Sats <sup>b</sup>	Tot Sat <sup>c</sup>
Westar 7.0	3.9	1.9	67.5	17.6	7.4	5.9	
15 W13097.4 ( $M_3$ )	3.1	1.4	63.9	18.6	9.5	4.5	5.6
W13097.4 ( $M_4$ )	3.1	1.4	66.2	19.9	6.0	4.5	5.5
20 A144.1.9 ( $M_5$ )	2.9	1.4	64.3	20.7	7.3	4.4	5.3

<sup>a</sup>Letter and numbers up to second decimal point indicate the plant line. Number after second decimal point indicates an individual plant.

25 <sup>b</sup>Sat=FDA Saturates

<sup>c</sup>Tot Sat=Total Saturate Content

The  $M_5$  seed of ten self-pollinated A144.1 (ATCC 40813) plants averaged 3.1% palmitic acid and 4.7% FDA saturates. One selfed plant (A144.1.9) contained 2.9% palmitic acid and FDA saturates of 4.4%. Bulk seed analysis from open-pollinated (A144.1) plants at the  $M_5$  generation averaged 3.1% palmitic acid and 4.7% FDA saturates. The fatty acid composition of the bulked and individual A144.1 lines are summarized in Table IV.

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**TABLE IV**

Fatty Acid Composition of A144  
Low Palmitic Acid/Low FDA Saturate Line

5	Genotype <sup>a</sup>	Percent Fatty Acids					
		C <sub>16:0</sub>	C <sub>18:0</sub>	C <sub>18:1</sub>	C <sub>16:2</sub>	C <sub>18:3</sub>	Sats <sup>b</sup> Tot Sat <sup>c</sup>
	Individually Self-Pollinated Plants						
	A144.1.1	3.2	1.6	64.4	20.5	7.0	4.8 5.9
	A144.1.2	3.0	1.5	67.4	18.6	6.3	4.5 5.7
10	A144.1.3	3.6	1.8	61.4	22.4	7.5	5.2 6.6
	A144.1.4	3.2	1.5	64.6	20.9	6.7	4.7 5.8
	A144.1.5	3.3	1.7	60.0	23.9	7.9	5.0 6.1
	A144.1.6	3.1	1.4	67.3	17.8	6.5	4.6 5.2
	A144.1.7	3.1	1.6	67.7	17.4	6.5	4.8 5.4
15	A144.1.8	3.1	1.8	66.9	18.7	6.1	4.9 5.4
	A144.1.9	2.9	1.4	64.3	20.7	7.3	4.4 5.3
	A144.1.10	3.1	1.5	62.5	20.4	7.7	4.6 5.6
	Average of Individually Self-Pollinated Plants						
	A144.1.1-10	3.1	1.6	64.8	20.1	6.9	4.7 5.7
20	Bulk Analysis of Open-Pollinated Plants						
	A144.1B	3.1	1.6	64.8	19.4	7.8	4.7 5.7

<sup>a</sup>Letter and numbers up to second decimal point indicate the plant line. Number after second decimal point indicates an individual plant.

<sup>b</sup>Sat=FDA Saturates

<sup>c</sup>Tot Sat=Total Saturate Content

These reduced levels have remained stable to the M<sub>7</sub> generations in both greenhouse and field conditions.

30 These reduced levels have remained stable to the M<sub>7</sub> generation in multiple location field trails. Over all locations, the self-pollinated plants (A144) averaged 2.9% palmitic acid and FDA saturates of 4.6%. The fatty

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acid composition of the A144 lines for each Idaho location are summarized in Table V. In the multiple location replicated trial the yield of A144 was not significantly different in yield from the parent cultivar Westar. By means of seed mutagenesis, the level of saturated fatty acids of canola (*B. napus*) was reduced from 5.9% to 4.6%. The palmitic acid content was reduced from 3.9% to 2.9%.

**TABLE V**

10 Fatty Acid Composition of a Mutant Low Palmitic Acid/Low FDA Saturate Canola Line at  
Different Field Locations in Idaho

Trial	Location	Percent Fatty Acids						
		C <sub>16:0</sub>	C <sub>18:0</sub>	C <sub>18:1</sub>	C <sub>18:2</sub>	C <sub>18:3</sub>	Sats	Tot Sats
15	Burley	2.9	1.3	62.3	20.6	10.3	4.2	5.0
	Tetonia	2.9	1.7	59.7	21.0	11.2	4.6	5.7
	Lamont	3.1	1.8	63.2	19.5	9.0	4.9	5.9
20	Shelley	2.8	1.9	64.5	18.8	8.8	4.7	5.9

To determine the genetic relationship of the palmitic acid mutation in A144 (C<sub>16:0</sub> - 3.0%, C<sub>18:0</sub> - 1.5%, C<sub>18:1</sub> - 67.4%, C<sub>18:2</sub> - 18.6%, C<sub>18:3</sub> - 6.3%) to other fatty acid mutations it was crossed to A129 a mutant high oleic acid (C<sub>16:0</sub> - 3.8%, C<sub>18:0</sub> - 2.3%, C<sub>18:1</sub> - 75.6%, C<sub>18:2</sub> - 9.5%, C<sub>18:3</sub> - 4.9%). Over 570 dihaploid progeny produced from the F<sub>1</sub> hybrid were harvested and analyzed for fatty acid composition. The results of the progeny analysis are summarized in Table VB. Independent segregation of the palmitic traits was observed which demonstrates that the genetic control of palmitic acid in A144 is different from the high oleic acid mutation in A129.

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TABLE VBGenetic Studies of Dihaploid Progeny of A144 X A129

	Genotype	C <sub>16:0</sub> Content (%)	Frequency	
			Observed	Expected
5	p-p-p2-p2-	3.0%	162	143
	p+p-p2-p2-	3.4%	236	286
	p+p-p2+p2+	3.8%	175	143

EXAMPLE 2

10 An additional low FDA saturate line, designated A149.3 (ATCC 40814), was also produced by the method of Example 1. A 50-seed bulk analysis of this line showed the following fatty acid composition: C<sub>16:0</sub> - 3.6%, C<sub>18:0</sub> - 1.4%, C<sub>18:1</sub> - 65.5%, C<sub>18:2</sub> - 18.3%, C<sub>18:3</sub> - 8.2%, FDA Sats -  
 15 5.0%, Total Sats - 5.9%. This line has also stably maintained its mutant fatty acid composition to the M<sub>5</sub> generation. In a multiple location replicated trial the yield of A149 was not significantly different in yield from the parent cultivar Westar.

EXAMPLE 3

20 An additional low palmitic acid and low FDA saturate line, designated M3094.4 (ATCC 75023), was also produced by the method of Example 1. A 10-seed bulk analysis of this line showed the following fatty acid  
 25 composition: C<sub>16:0</sub> - 2.7%, C<sub>18:0</sub> - 1.6%, C<sub>18:1</sub> - 66.6%, C<sub>18:2</sub> - 20.0%, C<sub>18:3</sub> - 6.1%, C<sub>20:1</sub> - 1.4%, C<sub>22:1</sub> - 0.0%, FDA Saturate - 4.3%, Total Saturates - 5.2%. This line has stably maintained its mutant fatty acid composition to the M<sub>5</sub> generation. In a single replicated trial the yield of  
 30 M3094 was not significantly different in yield from the parent cultivar.

M3094.4 was crossed to A144, a low palmitic acid mutation (Example 1) for allelism studies. Fatty acid composition of the F<sub>2</sub> seed showed the two lines to be

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allelic. The mutational events in A144 and M3094, although different in origin, are in the same gene.

#### EXAMPLE 4

In the studies of Example 1, at the  $M_1$  generation, 470 lines exceed the upper statistical threshold for palmitic acid ( $\geq 4.3\%$ ). One  $M_1$  line, W14538.6, contained 9.2% palmitic acid. Selfed progenies of this line, since designated M3007.4 (ATCC 75022), continued to exceed to the upper statistical threshold for high palmitic acid at both the  $M_4$  and  $M_5$  generations with palmitic acid levels of 11.7% and 9.1%, respectively. The fatty acid composition of this high palmitic acid mutant, which was stable to the  $M_5$  generation under both field and greenhouse conditions, is summarized in Table VI.

TABLE VI

Fatty Acid Composition of a High Palmitic Acid Canola Line Produced by Seed Mutagenesis

Genotype	Percent Fatty Acids					
	C <sub>16:0</sub>	C <sub>18:0</sub>	C <sub>18:1</sub>	C <sub>18:2</sub>	C <sub>18:3</sub>	Sats*
Westar	3.9	1.9	67.5	17.6	7.4	7.0
W114538.6 ( $M_1$ )	8.6	1.6	56.4	20.3	9.5	10.2
M3007.2 ( $M_4$ )	11.7	2.1	57.2	18.2	5.1	13.9
M3007.4 ( $M_5$ )	9.1	1.4	63.3	13.7	5.5	12.7

\*Sats=Total Saturate Content

To determine the genetic relationship of the high palmitic mutation in M3007.4 to the low palmitic mutation in A144 (Example 1) crosses were made. The  $F_2$  progeny were analyzed for fatty acid composition. The data presented in Table VIB shows the high palmitic group (C<sub>16:0</sub>

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> 7.0%) makes up one-quarter of the total population analyzed. The high palmitic acid mutation was controlled by one single gene mutation.

**TABLE VIB**Genetic Studies of M3007 X A144

5

		<u>Frequency</u>	
<u>Genotype</u>	<u>C<sub>16:0</sub> Content (%)</u>	<u>Observed</u>	<u>Expected</u>
p-p-/p-hp-	<7.0	151	142
10 hp-hp-	>7.0	39	47

An additional M<sub>1</sub> line, W4773.7, contained 4.5% palmitic acid. Selfed progenies of this line, since designated A200.7 (ATCC 40816), continued to exceed the upper statistical threshold for high palmitic acid in both the M<sub>1</sub> and M<sub>2</sub> generations with palmitic acid levels of 6.3% and 6.0%, respectively. The fatty acid composition of this high palmitic acid mutant, which was stable to the M<sub>5</sub> generation under both field and greenhouse conditions, is summarized in Table VII.

20

**TABLE VII**Fatty Acid Composition of a High Palmitic Acid Canola Line Produced by Seed Mutagenesis

		<u>Percent Fatty Acids</u>				
<u>Genotype</u>	<u>C<sub>16:0</sub></u>	<u>C<sub>18:0</sub></u>	<u>C<sub>18:1</sub></u>	<u>C<sub>18:2</sub></u>	<u>C<sub>18:3</sub></u>	<u>Sats*</u>
25 Westar	3.9	1.9	67.5	17.6	7.4	7.0
W4773.7 (M <sub>3</sub> )	4.5	2.9	63.5	19.9	7.1	9.3
M4773.7.7 (M <sub>4</sub> )	6.3	2.6	59.3	20.5	5.6	10.8
30 A200.7.7 (M <sub>5</sub> )	6.0	1.9	60.2	20.4	7.3	9.4
*Sats=Total Saturate Content						

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EXAMPLE 5Selection of Low Stearic Acid Canola Lines

In the studies of Example 1, at the  $M_1$  generation, 42 lines exceeded the lower statistical threshold for stearic acid (<1.4%). Line W14859.6 had 1.3% stearic acid. At the  $M_2$  generation, its selfed progeny (M3052.1) continued to fall within the lower statistical threshold for  $C_{18:0}$  with 0.8% stearic acid. The fatty acid composition of this low stearic acid mutant, which was stable under both field and greenhouse conditions is summarized in Table VIII. In a single location replicated yield trial M3052.1 was not significantly different in yield from the parent cultivar Westar.

TABLE VIII

Fatty Acid Composition of a Low  
Stearic Acid Canola Line Produced by Seed Mutagenesis

		<u>Percent Fatty Acids</u>					
<u>Genotype</u>		<u>C<sub>16:0</sub></u>	<u>C<sub>18:0</sub></u>	<u>C<sub>18:1</sub></u>	<u>C<sub>18:2</sub></u>	<u>C<sub>22:1</sub></u>	<u>Sats</u>
	Westar	3.9	1.9	67.5	17.6	7.4	5.9
20	W14859.6 (M <sub>3</sub> )	5.3	1.3	56.1	23.7	9.6	7.5
	M3052.1 (M <sub>4</sub> )	4.9	0.9	58.9	22.7	9.3	5.8
	M3052.6 (M <sub>5</sub> )	4.4	0.8	62.1	21.2	7.9	5.2
25							

To determine the genetic relationship of the low stearic acid mutation of M3052.1 to other fatty acid mutations it was crossed to the low palmitic acid mutation A144 (Example 1). Seed from over 300 dihaploid progeny were harvested and analyzed for fatty acid composition. The results are summarized in Table VIIIB. Independent segregation of the palmitic acid and stearic acid traits was observed. The low stearic acid mutation



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was genetically different from the low palmitic acid mutations found in A144 and M3094.

**TABLE VIIIB**  
**Genetic Studies of M3052 X A144**

5	Genotype	C <sub>16:0</sub> + C <sub>18:0</sub> Content (%)	Frequency	
			Observed	Expected
	p-p-s-s-	<4.9%	87	77
10	p-p-s-s-/p+p+s-s-	4.0%<X<5.6%	152	154
	p+p+s+s+	>5.6%	70	77

An additional M<sub>5</sub> line, M3051.10, contained 0.9% and 1.1% stearic acid in the greenhouse and field respectively. A ten-seed analysis of this line showed the following fatty acid composition: C<sub>16:0</sub> - 3.9%, C<sub>18:0</sub> - 1.1%, C<sub>18:1</sub> - 61.7%, C<sub>18:2</sub> - 23.0%, C<sub>18:3</sub> - 7.6%, FDA saturates - 5.0%, Total Saturates - 5.8%. In a single location replicated yield trial M3051.10 was not significantly different in yield from the parent cultivar Westar. M3051.10 was crossed to M3052.1 for allelism studies. Fatty acid composition of the F<sub>2</sub> seed showed the two lines to be allelic. The mutational events in M3051.10 and M3052.1 although different in origin were in the same gene.

25 An additional M<sub>5</sub> line, M3054.7, contained 1.0% and 1.3% stearic acid in the greenhouse and field respectively. A ten-seed analysis of this line showed the following fatty acid composition: C<sub>16:0</sub> - 4.0%, C<sub>18:0</sub> - 1.0%, C<sub>18:1</sub> - 66.5%, C<sub>18:2</sub> - 18.4%, C<sub>18:3</sub> - 7.2%, saturates - 5.0%, Total Saturates - 6.1%. In a single location replicated yield trial M3054.7 was not significantly different in yield from the parent cultivar Westar. M3054.7 was crossed to M3052.1 for allelism studies.

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Fatty acid composition of the  $F_2$  seed showed the two lines to be allelic. The mutational events in M3054.7, M3051.10 and M3052.1 although different in origin were in the same gene.

5

EXAMPLE 6High Oleic Acid Canola Lines

In the studies of Example 1, at the  $M_1$  generation, 31 lines exceeded the upper statistical threshold for oleic acid ( $\geq 71.0\%$ ). Line W7608.3 had 71.2% oleic acid. At the  $M_1$  generation, its selfed progeny (W7608.3.5, since designated A129.5) continued to exceed the upper statistical threshold for  $C_{18:1}$  with 78.8% oleic acid.  $M_2$  seed of five self-pollinated plants of line A129.5 (ATCC 40811) averaged 75.0% oleic acid. A single plant selection, A129.5.3 had 75.6% oleic acid. The fatty acid composition of this high oleic acid mutant, which was stable under both field and greenhouse conditions to the  $M_2$  generation, is summarized in Table IX. This line also stably maintained its mutant fatty acid composition to the  $M_2$  generation in field trials in multiple locations. Over all locations the self-pollinated plants (A129) averaged 78.3% oleic acid. The fatty acid composition of the A129 for each Idaho trial location are summarized in Table X. In multiple location replicated yield trials, A129 was not significantly different in yield from the parent cultivar Westar.

The canola oil of A129, after commercial processing, was found to have superior oxidative stability compared to Westar when measured by the Accelerated Oxygen Method (AOM), American Oil Chemists' Society Official Method Cd 12-57 for fat stability; Active Oxygen Method (revised 1989). The AOM of Westar was 18 AOM hours and for A129 was 30 AOM hours.

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**TABLE IX**

Fatty Acid Composition of a High  
Oleic Acid Canola Line Produced by Seed Mutagenesis

Percent Fatty Acids

5	Genotype	Percent Fatty Acids					Sats
		C <sub>16:0</sub>	C <sub>18:0</sub>	C <sub>18:1</sub>	C <sub>18:2</sub>	C <sub>18:3</sub>	
	Westar	3.9	1.9	67.5	17.6	7.4	7.0
	W7608.3 (M <sub>3</sub> )	3.9	2.4	71.2	12.7	6.1	7.6
	W7608.3.5 (M <sub>4</sub> )	3.9	2.0	78.8	7.7	3.9	7.3
10	A129.5.3 (M <sub>5</sub> )	3.8	2.3	75.6	9.5	4.9	7.6

Sats=Total Saturate Content

**TABLE X**

15 Fatty Acid Composition of a Mutant High  
Oleic Acid Line at Different Field Locations in Idaho

Percent Fatty Acids

20	Location	Percent Fatty Acids					Sats
		C <sub>16:0</sub>	C <sub>18:0</sub>	C <sub>18:1</sub>	C <sub>18:2</sub>	C <sub>18:3</sub>	
	Burley	3.3	2.1	77.5	8.1	6.0	6.5
	Tetonia	3.5	3.4	77.8	6.5	4.7	8.5
	Lamont	3.4	1.9	77.8	7.4	6.5	6.3
	Shelley	3.3	2.6	80.0	5.7	4.5	7.7

Sats=Total Saturate Content

The genetic relationship of the high oleic acid  
25 mutation A129 to other oleic desaturases was demonstrated  
in crosses made to commercial canola cultivars and a low  
linolenic acid mutation. A129 was crossed to the  
commercial cultivar Global (C<sub>16:0</sub> - 4.5%, C<sub>18:0</sub> - 1.5%, C<sub>18:1</sub>  
- 62.9%, C<sub>18:2</sub> - 20.0%, C<sub>18:3</sub> - 7.3%). Approximately 200 F<sub>2</sub>  
30 individuals were analyzed for fatty acid composition.  
The results are summarized in Table XB. The segregation  
fit 1:2:1 ratio suggesting a single co-dominant gene

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controlled the inheritance of the high oleic acid phenotype.

**TABLE XB**Genetic Studies of A129 X Global

5

Frequency

	Genotype	C <sub>18:3</sub> Content (%)	Frequency	
			Observed	Expected
	od-od-	77.3	43	47
10	od-od+	71.7	106	94
	od+od+	66.1	49	47

A cross between A129 and IMC 01, a low linolenic acid variety (C<sub>16:0</sub> - 4.1%, C<sub>18:0</sub> - 1.9%, C<sub>18:1</sub> - 66.4%, C<sub>18:2</sub> - 18.1%, C<sub>18:3</sub> - 5.7%), was made to determine the inheritance of the oleic acid desaturase and linoleic acid desaturase. In the F<sub>1</sub> hybrids both the oleic acid and linoleic acid desaturase genes approached the mid-parent values indicating a co-dominant gene actions. Fatty acid analysis of the F<sub>2</sub> individuals confirmed a 1:2:1:2:4:2:1:2:1 segregation of two independent, co-dominant genes (Table XC). A line was selected from the cross of A129 and IMC01 and designated as IMC130 (ATCC deposit no. 75446) as described in U.S. Patent Application No. 08/425,108, incorporated herein by reference.

**TABLE XC**Genetic Studies of A129 X IMC 01

Frequency

	Genotype	Ratio	Frequency	
			Observed	Expected
30	od-od-ld-ld-	1	11	12
	od-od-ld-ld+	2	30	24
	od-od-ld+ld+	1	10	12
	od-od+ld-ld-	2	25	24
	od-od+ld-ld+	4	54	47
35	od-od+ld+ld+	2	18	24
	od+od+ld-ld-	1	7	12
	od+od+ld-ld+	2	25	24
	od+od+ld+ld+	1	8	12

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An additional high oleic acid line, designated A128.3, was also produced by the disclosed method. A 50-seed bulk analysis of this line showed the following fatty acid composition:  $C_{16:0}$  - 3.5%,  $C_{18:0}$  - 1.8%,  $C_{18:1}$  - 77.3%,  $C_{18:2}$  - 9.0%,  $C_{18:3}$  - 5.6%, FDA Sats - 5.3%, Total Sats - 6.4%. This line also stably maintained its mutant fatty acid composition to the  $M_3$  generation. In multiple locations replicated yield trials, A128 was not significantly different in yield from the parent cultivar Westar.

A129 was crossed to A128.3 for allelism studies. Fatty acid composition of the  $F_2$  seed showed the two lines to be allelic. The mutational events in A129 and A128.3 although different in origin were in the same gene.

An additional high oleic acid line, designated M3028.-10 (ATCC 75026), was also produced by the disclosed method in Example 1. A 10-seed bulk analysis of this line showed the following fatty acid composition:  $C_{16:0}$  - 3.5%,  $C_{18:0}$  - 1.8%,  $C_{18:1}$  - 77.3%,  $C_{18:2}$  - 9.0%,  $C_{18:3}$  - 5.6%, FDA Saturates - 5.3%, Total Saturates - 6.4%. In a single location replicated yield trial M3028.10 was not significantly different in yield from the parent cultivar Westar.

#### EXAMPLE 7

##### Low Linoleic Acid Canola

In the studies of Example 1, at the  $M_1$  generation, 80 lines exceeded the lower statistical threshold for linoleic acid ( $\leq 13.2\%$ ). Line W12638.8 had 9.4% linoleic acid. At the  $M_4$  and  $M_5$  generations, its selfed progenies [W12638.8, since designated A133.1 (ATCC 40812)] continued to exceed the statistical threshold for low  $C_{18:2}$  with linoleic acid levels of 10.2% and 8.4%, respectively. The fatty acid composition of this low linoleic acid mutant, which was stable to the  $M_5$  generation under both field and greenhouse conditions, is

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summarized in Table XI. In multiple location replicated yield trials, A133 was not significantly different in yield from the parent cultivar Westar. An additional low linoleic acid line, designated M3062.8 (ATCC 75025), was also produced by the disclosed method. A 10-seed bulk analysis of this line showed the following fatty acid composition: C<sub>16:0</sub> - 3.8%, C<sub>18:0</sub> - 2.3%, C<sub>18:1</sub> - 77.1%, C<sub>18:2</sub> - 8.9%, C<sub>18:3</sub> - 4.3%, FDA Sats-6.1%. This line has also stably maintained its mutant fatty acid composition in the field and greenhouse.

**TABLE XI**

Fatty Acid Composition of a Low  
Linoleic Acid Canola Line Produced by Seed Mutagenesis

		Percent Fatty Acids					
15	Genotype	C <sub>16:0</sub>	C <sub>18:0</sub>	C <sub>18:1</sub>	C <sub>18:2</sub>	C <sub>18:3</sub>	Sats <sup>c</sup>
	Westar	3.9	1.9	67.5	17.6	7.4	7.0
	W12638.8 (M <sub>3</sub> )	3.9	2.3	75.0	9.4	6.1	7.5
	W12638.8.1 (M <sub>4</sub> )	4.1	1.7	74.6	10.2	5.9	7.1
20	A133.1.8 (M <sub>6</sub> )	3.8	2.0	77.7	8.4	5.0	7.0

<sup>a</sup>Letter and numbers up to second decimal point indicate the plant line. Number after second decimal point indicates an individual plant.

<sup>b</sup>Sats=Total Saturate Content

**EXAMPLE 8****Low Linolenic and Linoleic Acid Canola**

In the studies of Example 1, at the M<sub>3</sub> generation, 57 lines exceeded the lower statistical threshold for linolenic acid ( $\leq 5.3\%$ ). Line W14749.8 had 5.3% linolenic acid and 15.0% linoleic acid. At the M<sub>4</sub> and M<sub>5</sub>

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generations, its selfed progenies [W14749.8, since designated M3032 (ATCC 75021)] continued to exceed the statistical threshold for low  $C_{18:3}$  with linolenic acid levels of 2.7% and 2.3%, respectively, and for a low sum of linolenic and linoleic acids with totals of 11.8% and 12.5% respectively. The fatty acid composition of this low linolenic acid plus linoleic acid mutant, which was stable to the  $M_5$  generation under both field and greenhouse conditions, is summarized in Table XII. In a single location replicated yield trial M3032 was not significantly different in yield from the parent cultivar (Westar).

**TABLE XII**

Fatty Acid Composition of a Low  
Linolenic Acid Canola Line Produced by Seed Mutagenesis  
 Percent Fatty Acids

Genotype	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	Sats
Westar	3.9	1.9	67.5	17.6	7.4	7.0
W14749.8 ( $M_3$ )	4.0	2.5	69.4	15.0	5.3	6.5
M3032.8 ( $M_4$ )	3.9	2.4	77.9	9.1	2.7	6.4
M3032.1 ( $M_5$ )	3.5	2.8	80.0	10.2	2.3	6.5
Sats=Total Saturate Content						

**EXAMPLE 9**

The high oleic acid mutation of A129 was introduced into different genetic backgrounds by crossing and selecting for fatty acid and agronomic characteristics. A129 (now renamed IMC 129) was crossed to Legend, a commercial spring *Brassica napus* variety. Legend has the following fatty acid composition:  $C_{16:0}$  - 3.8%,  $C_{18:0}$  - 2.1%,  $C_{18:1}$  - 63.1%,  $C_{18:2}$  - 17.8%,  $C_{18:3}$  - 9.3%.

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The cross and progeny resulting from were coded as 89B60303.

The  $F_1$  seed resulting from the cross was planted in the greenhouse and self-pollinated to produce  $F_2$  seed.

5 The  $F_2$  seed was planted in the field for evaluation. Individual plants were selected in the field for agronomic characteristics. At maturity, the  $F_2$  seed was harvested from each selected plant and analyzed for fatty acid composition.

10 Individuals which had fatty acid profiles similar to the high oleic acid parent (IMC 129) were advanced back to the field. Seeds ( $F_3$ ) of selected individuals were planted in the field as selfing rows and in plots for preliminary yield and agronomic evaluations. At  
15 flowering the  $F_3$  plants in the selfing rows were self-pollinated. At maturity the  $F_3$  seed was harvested from individual plants to determine fatty acid composition. Yield of the individual selections was determined from the harvested plots.

20 Based on fatty acid composition of the individual plants and yield and agronomic characteristics of the plots  $F_4$  lines were selected and advanced to the next generation in the greenhouse. Five plants from each selected line were self-pollinated. At maturity the  $F_4$   
25 seed was harvested from each and analyzed for fatty acid composition.

The  $F_4$  line with the highest oleic fatty profile was advanced to the field as a selfing row. The remaining  $F_4$  seed from the five plants was bulked together  
30 for planting the yield plots in the field. At flowering, the  $F_5$  plants in each selfing-row were self-pollinated. At maturity the  $F_5$  self-pollinated seed was harvest from the selfing row to determine fatty acid composition and select for the high oleic acid trait. Yield of the



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individual selections was determined from the harvested plots.

Fifteen  $F_2$  lines having the high oleic fatty profile of IMC 129 and the desired agronomic characteristics were advanced to the greenhouse to increase seed for field trialing. At flowering the  $F_2$  plants were self-pollinated. At maturity the  $F_2$  seed was harvested and analyzed for fatty acid composition. Three  $F_2$  seed lines which had fatty acid profiles most similar to IMC 129 (Table XIII) were selected and planted in the field as selfing rows, the remaining seed was bulked together for yield trialing. The high oleic fatty acid profile of IMC 129 was maintained through seven generations of selection for fatty acid and agronomic traits in an agronomic background of *Brassica napus* which was different from the parental lines. Thus, the genetic trait from IMC 129 for high oleic acid can be used in the development of new high oleic *Brassica napus* varieties.

**TABLE XIII**

Fatty Acid Composition of Advanced Breeding Generation with High Oleic Acid Trait (IMC 129 X Legend)

F <sub>2</sub> Selections of 89B60303	Fatty Acid Composition(%)				
	C <sub>16:0</sub>	C <sub>18:0</sub>	C <sub>18:1</sub>	C <sub>18:2</sub>	C <sub>18:3</sub>
93.06194	3.8	1.6	78.3	7.7	4.4
93.06196	4.0	2.8	77.3	6.8	3.4
93.06198	3.7	2.2	78.0	7.4	4.2

The high oleic acid trait of IMC 129 was also introduced into a different genetic background by combining crossing and selection methods with the generation of dihaploid populations from the microspores of the  $F_1$  hybrids. IMC 129 was crossed to Hyola 41, a commercial spring *Brassica napus* variety. Hyola 41 has the following fatty acid composition: C<sub>16:0</sub> - 3.8%, C<sub>18:0</sub> -

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2.7%,  $C_{18:1}$  - 64.9%,  $C_{18:2}$  - 16.2%,  $C_{19}$  - 9.1%. The cross and progeny resulting from the cross were labeled 90DU.146.

The  $F_1$  seed was planted from the cross and a dihaploid ( $DH_1$ ) population was made from the  $F_1$  microspores using standard procedures for *Brassica napus*. Each  $DH_1$  plant was self-pollinated at flowering to produce  $DH_2$  seed. At maturity the  $DH_1$  seed was harvested and analyzed for fatty acid composition.  $DH_1$  individuals which expressed the high oleic fatty acid profile of IMC 129 were advanced to the next generation in the greenhouse. For each individual selected five  $DH_1$  seeds were planted. At flowering the  $DH_2$  plants were self-pollinated. At maturity the  $DH_2$  seed was harvested and analyzed for fatty acid composition. The  $DH_2$  seed which was similar in fatty acid composition to the IMC 129 parent was advanced to the field as a selfing row. The remaining  $DH_2$  seed of that group was bulked and planted in plots to determine yield and agronomic characteristics of the line. At flowering individual  $DH_1$  plants in the selfing row were self-pollinated. At maturity the  $DH_2$  seed was harvested from the individual plants to determine fatty acid composition. Yield of the selections was determined from the harvested plots. Based on fatty acid composition, yield and agronomic characteristics selections were advanced to the next generation in the greenhouse. The  $DH_2$  seed produced in the greenhouse by self-pollination was analyzed for fatty acid composition. Individuals which were similar to the fatty acid composition of the IMC 129 parent were advanced to the field to test for fatty acid stability and yield evaluation. The harvested  $DH_2$  seed from six locations maintained the fatty acid profile of the IMC 129 parent (Table XIV).

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TABLE XIV

Fatty Acid Composition of Advanced Dihaploid Breeding  
Generation with High Oleic Acid Trait  
(IMC 129 X Hyola41)

5	DH5 of 90DU.146 at Multiple Locations	Fatty Acid Composition(%)				
		C <sub>16:0</sub>	C <sub>18:0</sub>	C <sub>18:1</sub>	C <sub>18:2</sub>	C <sub>18:3</sub>
	Aberdeen	3.7	2.6	75.4	8.1	7.2
10	Blackfoot	3.3	2.4	75.5	8.8	7.5
	Idaho Falls	3.7	3.1	75.0	7.5	8.1
	Rexberg	3.9	3.7	75.3	7.0	6.5
	Swan Valley	3.5	3.4	74.5	7.0	7.3
	Lamont	3.9	2.8	72.0	10.1	8.4

15

EXAMPLE 10Canola Lines Q508 and Q4275

Seeds of the *B. napus* line IMC-129 were  
mutagenized with methyl N-nitrosoguanidine (MNNG). The  
MNNG treatment consisted of three parts: pre-soak,  
20 mutagen application, and wash. A 0.05M Sorenson's  
phosphate buffer was used to maintain pre-soak and  
mutagen treatment pH at 6.1. Two hundred seeds were  
treated at one time on filter paper (Whatman #3M) in a  
petri dish (100mm x 15mm). The seeds were pre-soaked in  
25 15 mls of 0.05M Sorenson's buffer, pH 6.1, under  
continued agitation for two hours. At the end of the  
pre-soak period, the buffer was removed from the plate.

A 10mM concentration of MNNG in 0.05M Sorenson's  
buffer, pH 6.1, was prepared prior to use. Fifteen ml of  
30 10m MNNG was added to the seeds in each plate. The seeds  
were incubated at 22°C±3°C in the dark under constant  
agitation for four (4) hours. At the end of the  
incubation period, the mutagen solution was removed.

The seeds were washed with three changes of  
35 distilled water at 10 minute intervals. The fourth wash

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was for thirty minutes. This treatment regime produced an LD60 population.

Treated seeds were planted in standard greenhouse potting soil and placed into an environmentally  
5 controlled greenhouse. The plants were grown under sixteen hours of light. At flowering, the racemes were bagged to produce selfed seed. At maturity, the M2 seed was harvested. Each M2 line was given an identifying number. The entire MNNG-treated seed population was  
10 designated as the Q series.

Harvested M2 seeds was planted in the greenhouse. The growth conditions were maintained as previously described. The racemes were bagged at flowering for selfing. At maturity, the selfed M3 seed was harvested  
15 and analyzed for fatty acid composition. For each M3 seed line, approximately 10-15 seeds were analyzed in bulk as described in Example 1.

High oleic-low linoleic M3 lines were selected from the M3 population using a cutoff of >82% oleic acid  
20 and <5.0% linoleic. From the first 1600 M3 lines screened for fatty acid composition, Q508 was identified. The Q508 M3 generation was advanced to the M4 generation in the greenhouse. Table XV shows the fatty acid composition of Q508 and IMC 129. The M4 selfed seed  
25 maintained the selected high oleic-low linoleic acid phenotype (Table XVI).

**TABLE XV**

Fatty Acid Composition of A129 and High  
Oleic Acid M3 Mutant Q508

30 Line #	16:0	18:0	18:1	18:2	18:3
A129*	4.0	2.4	77.7	7.8	4.2
Q508	3.9	2.1	84.9	2.4	2.9

\*Fatty acid composition of A129 is the average of  
35 50 self-pollinated plants grown with the M3 population

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M<sub>1</sub> generation Q508 plants had poor agronomic qualities in the field compared to Westar. Typical plants were slow growing relative to Westar, lacked early vegetative vigor, were short in stature, tended to be chlorotic and had short pods. The yield of Q508 was very low compared to Westar.

The M<sub>1</sub> generation Q508 plants in the greenhouse tended to be reduced in vigor compared to Westar. However, Q508 yields in the greenhouse were greater than Q508 yields in the field.

**TABLE XVI**

Fatty Acid Composition of Seed Oil  
from Greenhouse-Grown Q508, IMC 129 and Westar.

Line	16:0	18:0	18:1	18:2	18:3	FDA Sats
IMC 129 <sup>a</sup>	4.0	2.4	77.7	7.8	4.2	6.4
Westar <sup>b</sup>	3.9	1.9	67.5	17.6	7.4	>5.8
Q508 <sup>c</sup>	3.9	2.1	84.9	2.4	2.9	6.0

<sup>a</sup>Average of 50 self-pollinated plants

<sup>b</sup>Data from Example 1

<sup>c</sup>Average of 50 self-pollinated plants

Nine other M<sub>4</sub> high-oleic low-linoleic lines were also identified: Q3603, Q3733, Q4249, Q6284, Q6601, Q6761, Q7415, Q4275, and Q6676. Some of these lines had good agronomic characteristics and an elevated oleic acid level in seeds of about 80% to about 84%.

Q4275 was crossed to the variety Cyclone. After selfing for seven generations, mature seed was harvested from 93GS34-179, a progeny line of the Q4275 Cyclone cross. Referring to Table XVII, fatty acid composition of a bulk seed sample shows that 93GS34 retained the seed fatty acid composition of Q4275. 93GS34-179 also maintained agronomically desirable characteristics.

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After more than seven generations of selfing of Q4275, plants of Q4275, IMC 129 and 93GS34 were field grown during the summer season. The selections were tested in 4 replicated plots (5 feet X 20 feet) in a randomized block design. Plants were open pollinated. No selfed seed was produced. Each plot was harvested at maturity, and a sample of the bulk harvested seed from each line was analyzed for fatty acid composition as described above. The fatty acid compositions of the 10 selected lines are shown in Table XVII.

**Table XVII**  
**Fatty Acid Composition of**  
**Field Grown IMC 129, Q4275 and 93GS34 Seeds**

Line	Fatty Acid Composition (%)					
	C <sub>16:0</sub>	C <sub>18:0</sub>	C <sub>18:1</sub>	C <sub>18:2</sub>	C <sub>19:3</sub>	FDA Sats
IMC 129	3.3	2.4	76.7	8.7	5.2	5.7
Q4275	3.7	3.1	82.1	4.0	3.5	6.8
93GS34-179	2.6	2.7	85.0	2.8	3.3	5.3

The results shown in Table XVII show that Q4275 maintained the selected high oleic - low linoleic acid phenotype under field conditions. The agronomic characteristics of Q4275 plants were superior to those of Q508.

M<sub>1</sub> generation Q508 plants were crossed to a dihaploid selection of Westar, with Westar serving as the female parent. The resulting F<sub>1</sub> seed was termed the 92EF population. About 126 F<sub>1</sub> individuals that appeared to have better agronomic characteristics than the Q508 parent were selected for selfing. A portion of the F<sub>2</sub> seed from such individuals was replanted in the field. Each F<sub>2</sub> plant was selfed and a portion of the resulting F<sub>3</sub> seed was analyzed for fatty acid composition. The content of oleic acid in F<sub>3</sub> seed ranged from 59 to 79%.

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No high oleic (>80%) individuals were recovered with good agronomic type.

A portion of the  $F_2$  seed of the 92EF population was planted in the greenhouse to analyze the genetics of the Q508 line.  $F_2$  seed was analyzed from 380  $F_2$  individuals. The  $C_{18:1}$  levels of  $F_2$  seed from the greenhouse experiment is depicted in Figure 1. The data were tested against the hypothesis that Q508 contains two mutant genes that are semi-dominant and additive: the original IMC 129 mutation as well as one additional mutation. The hypothesis also assumes that homozygous Q508 has greater than 85% oleic acid and homozygous Westar has 62-67% oleic acid. The possible genotypes at each gene in a cross of Q508 by Westar may be designated as:

AA = Westar Fad2<sup>a</sup>

BB = Westar Fad2<sup>b</sup>

aa = Q508 Fad2<sup>a</sup>

bb = Q508 Fad2<sup>b</sup>

Assuming independent segregation, a 1:4:6:4:1 ratio of phenotypes is expected. The phenotypes of heterozygous plants are assumed to be indistinguishable and, thus, the data were tested for fit to a 1:14:1 ratio of homozygous Westar: heterozygous plants: homozygous Q508.

Phenotypic	# of	
<u>Ratio</u>	<u>Westar Alleles</u>	<u>Genotype</u>
1	4	AABB (Westar)
4	3	AABb, AaBB, AABb, AaBB
6	2	AaBb, AAbb, AaBb, AaBb, aaBB, AaBb
4	1	Aabb, aaBb, Aabb, aaBb
1	0	aabb (Q508)

Using Chi-square analysis, the oleic acid data fit a 1:14:1 ratio. It was concluded that Q508 differs from

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Westar by two major genes that are semi-dominant and additive and that segregate independently. By comparison, the genotype of IMC 129 is aaBB.

The fatty acid composition of representative F<sub>3</sub> individuals having greater than 85% oleic acid in seed oil is shown in Table XVIII. The levels of saturated fatty acids are seen to be decreased in such plants, compared to Westar.

**TABLE XVIII**

10 92EF F<sub>3</sub> Individuals with >85% C<sub>18:1</sub> in Seed Oil

F3 Plant Identifier	Fatty Acid Composition (%)					
	C16:0	C18:0	C18:1	C18:2	C18:3	FDASA
+38068	3.401	1.582	85.452	2.134	3.615	4.983
+38156	3.388	1.379	85.434	2.143	3.701	4.767
15 +38171	3.588	1.511	85.289	2.367	3.425	5.099
+38181	3.75	1.16	85.312	2.968	3.819	4.977
+38182	3.529	0.985	85.905	2.614	3.926	4.56
+38191	3.364	1.039	85.737	2.869	4.039	4.459
+38196	3.557	1.182	85.054	2.962	4.252	4.739
20 +38202	3.554	1.105	86.091	2.651	3.721	4.713
+38220	3.093	1.16	86.421	1.931	3.514	4.314
+38236	3.308	1.349	85.425	2.37	3.605	4.718
+38408	3.617	1.607	85.34	2.33	3.562	5.224
+38427	3.494	1.454	85.924	2.206	3.289	4.948
25 +38533	3.64	1.319	85.962	2.715	3.516	4.959



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EXAMPLE 11Leaf and Root Fatty Acid Profiles of Canola  
Lines IMC-129, Q508, and Westar

Plants of Q508, IMC 129 and Westar were grown in  
5 the greenhouse. Mature leaves, primary expanding leaves,  
petioles and roots were harvested at the 6-8 leaf stage,  
frozen in liquid nitrogen and stored at -70°C. Lipid  
extracts were analyzed by GLC as described in Example 1.  
The fatty acid profile data are shown in Table XIX.

10 The data in Table XIX indicate that total leaf  
lipids in Q508 are higher in  $C_{18:1}$  content than the  $C_{18:2}$   
plus  $C_{18:3}$  content. The reverse is true for Westar and IMC  
129. The difference in total leaf lipids between Q508  
and IMC 129 is consistent with the hypothesis that a  
15 second Fad2 gene is mutated in Q508.

The  $C_{16:3}$  content in the total lipid fraction was  
about the same for all three lines, suggesting that the  
plastid FadC gene product was not affected by the Q508  
mutations. To confirm that the FadC gene was not  
20 mutated, chloroplast lipids were separated and analyzed.  
No changes in chloroplast  $C_{16:1}$ ,  $C_{16:2}$  or  $C_{16:3}$  fatty acids  
were detected in the three lines. The similarity in  
plastid leaf lipids among Q508, Westar and IMC 129 is  
consistent with the hypothesis that the second mutation  
25 in Q508 affects a microsomal Fad2 gene and not a plastid  
FadC gene.

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TABLE XIX

	MATURE LEAF			EXPANDING LEAF			PETIOLE			ROOT		
	West.	129	Q508	West.	129	Q508	West.	129	Q508	West.	129	Q508
16:0	12.1	11.9	10.1	16.4	16.1	11.3	21.7	23.5	11.9	21.1	21.9	12.0
16:1	0.8	0.6	1.1	0.7	0.6	1.1	1.0	1.1	1.4	-	-	-
16:2	2.3	2.2	2.0	2.8	3.1	2.8	1.8	2.2	1.8	-	-	-
16:3	14.7	15.0	14.0	6.3	5.4	6.9	5.7	4.6	5.7	-	-	-
18:0	2.2	1.6	1.2	2.5	2.8	1.5	3.7	4.0	1.6	3.6	2.9	2.5
18:1	2.8	4.9	16.7	3.8	8.3	38.0	4.9	12.9	46.5	3.5	6.1	68.8
18:2	12.6	11.5	6.8	13.3	13.8	4.9	20.7	18.1	5.2	28.0	33.4	4.4
18:3	50.6	50.3	46.0	54.2	50.0	33.5	40.4	33.2	25.1	43.8	38.7	12.3

10

EXAMPLE 12Sequences of Mutant and Wild-Type Delta-12 Fatty Acid  
Desaturases from *B. napus*

Primers specific for the FAD2 structural gene were used to clone the entire open reading frame (ORF) of the D and F 12-DES genes by reverse transcriptase polymerase chain reaction (RT-PCR). RNA from seeds of IMC 129, Q508 and Westar plants was isolated by standard methods and was used as template. The RT-amplified fragments were used for nucleotide sequence determination. The DNA sequence of each gene from each line was determined from both strands by standard dideoxy sequencing methods.

Sequence analysis revealed a G to A transversion at nucleotide 316 (from the translation initiation codon) of the D gene in both IMC 129 (SEQ ID NO:3) and Q508, compared to the sequence of Westar (SEQ ID NO:1). The transversion changes the codon at this position from GAG to AAG and results in a non-conservative substitution of glutamic acid, an acidic residue, for lysine a basic residue. The presence of the same mutation in both lines was expected since the Q508 line was derived from IMC 129. The same base change was also detected in Q508

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and IMC 129 when RNA from leaf tissue was used as template.

The G to A mutation at nucleotide 316 was confirmed by sequencing several independent clones containing fragments amplified directly from genomic DNA of IMC 129 and Westar. These results eliminated the possibility of a rare mutation introduced during reverse transcription and PCR in the RT-PCR protocol. It was concluded that the IMC 129 mutant is due to a single base transversion at nucleotide 316 in the coding region of the D gene of rapeseed microsomal delta 12-desaturase.

A single base transition from T to A at nucleotide 515 of the F gene was detected in Q508 compared to the Westar sequence. The mutation changes the codon at this position from CTC to CAC, resulting in the non-conservative substitution of a non-polar residue, leucine, for a polar residue, histidine, in the resulting gene product. No mutations were found in the F gene sequence of IMC 129 compared to the F gene sequence of Westar.

These data support the conclusion that a mutation in a delta-12 desaturase gene sequence results in alterations in the fatty acid profile of plants containing such a mutated gene. Moreover, the data show that when a plant line or species contains two delta-12 desaturase loci, the fatty acid profile of an individual having two mutated loci differs from the fatty acid profile of an individual having one mutated locus.

The mutation in the D gene of IMC 129 and Q508 mapped to a region having a conserved amino acid motif (His-Xaa-Xaa-Xaa-His) found in cloned delta-12 and delta-15 membrane bound-desaturases (Table XX).

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Table XX

Alignment of Amino Acid Sequences  
of Cloned Canola Membrane Bound-Desaturases

	Desaturase Gene	Sequence <sup>a</sup>	Position
5	Canola-fad2-D (mutant)	AHKCGH	109-114
	Canola-Fad2-D	AHECGH	109-114
	Canola-Fad2-F	AHECGH	109-114
	Canola-FadC	<u>GHDC</u> AH	170-175
	Canola-fad3 (mutant)	<u>GHK</u> CGH	94-99
10	Canola-Fad3	<u>GHDC</u> CGH	94-99
	Canola-FadD	<u>GHDC</u> CGH	125-130

(FadD = Plastid delta 15, Fad3 = Microsomal delta-15),

(FadC = Plastid delta-12, Fad2 = Microsomal delta-12)

<sup>a</sup> One letter amino acid code; conservative substitutions  
15 are underlined; non-conservative substitutions are in  
bold.

EXAMPLE 13

Transcription and Translation of Microsomal Delta-12  
Fatty Acid Desaturases

20 Transcription in vivo was analyzed by RT-PCR  
analysis of stage II and stage III developing seeds and  
leaf tissue. The primers used to specifically amplify  
12-DES F gene RNA from the indicated tissues were sense  
primer 5'-GGATATGATGATGGTGAAAGA-3' and antisense primer  
25 5'-TCTTTCACCATCATCATATCC-3'. The primers used to  
specifically amplify 12-DES D gene RNA from the indicated  
tissues were sense primer 5'-GTTATGAAGCAAAGAAGAAAC-3' and  
antisense primer 5'-GTTTCTTCTTTGCTTCATAAC-3'. The  
results indicated that mRNA of both the D and F gene was  
30 expressed in seed and leaf tissues of IMC 129, Q508 and  
wild type Westar plants.

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In vitro transcription and translation analysis showed that a peptide of about 46 kD was made. This is the expected size of both the D gene product and the F gene product, based on sum of the deduced amino acid sequence of each gene and the cotranslational addition of a microsomal membrane peptide.

These results rule out the possibility that non-sense or frameshift mutations, resulting in a truncated polypeptide gene product, are present in either the mutant D gene or the mutant F gene. The data, in conjunction with the data of Example 12, support the conclusion that the mutations in Q508 and IMC 129 are in delta-12 fatty acid desaturase structural genes encoding desaturase enzymes, rather than in regulatory genes.

15

EXAMPLE 14Development of Gene-Specific PCR Markers

Based on the single base change in the mutant D gene of IMC 129 described in above, two 5' PCR primers were designed. The nucleotide sequence of the primers differed only in the base (G for Westar and A for IMC 129) at the 3' end. The primers allow one to distinguish between mutant fad2-D and wild-type Fad2-D alleles in a DNA-based PCR assay. Since there is only a single base difference in the 5' PCR primers, the PCR assay is very sensitive to the PCR conditions such as annealing temperature, cycle number, amount, and purity of DNA templates used. Assay conditions have been established that distinguish between the mutant gene and the wild type gene using genomic DNA from IMC 129 and wild type plants as templates. Conditions may be further optimized by varying PCR parameters, particularly with variable crude DNA samples. A PCR assay distinguishing the single base mutation in IMC 129 from the wild type gene along with fatty acid composition analysis provides a means to

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simplify segregation and selection analysis of genetic crosses involving plants having a delta-12 fatty acid desaturase mutation.

#### EXAMPLE 15

##### 5     Transformation with Mutant and Wild Type Fad3 Genes

*B. napus* cultivar Westar was transformed with mutant and wild type Fad3 genes to demonstrate that the mutant Fad3 gene for canola cytoplasmic linoleic desaturase 15-DES is nonfunctional. Transformation and  
10 regeneration were performed using disarmed *Agrobacterium tumefaciens* essentially following the procedure described in WO 94/11516.

Two disarmed *Agrobacterium* strains were engineered, each containing a Ti plasmid having the  
15 appropriate gene linked to a seed-specific promoter and a corresponding termination sequence. The first plasmid, pIMC110, was prepared by inserting into a disarmed Ti vector the full length wild type Fad3 gene in sense orientation (nucleotides 208 to 1336 of SEQ ID 6 in WO  
20 93/11245), flanked by a napin promoter sequence positioned 5' to the Fad3 gene and a napin termination sequence positioned 3' to the Fad3 gene. The rapeseed napin promoter is described in EP 0255378.

The second plasmid, pIMC205, was prepared by  
25 inserting a mutated Fad3 gene in sense orientation into a disarmed Ti vector. The mutant sequence contained mutations at nucleotides 411 and 413 of the microsomal Fad3 gene described in WO93/11245, thus changing the sequence for codon 96 from GAC to AAG. The amino acid at  
30 codon 96 of the gene product was thereby changed from aspartic acid to lysine. See Table XX. A bean (*Phaseolus vulgaris*) phaseolin (7S seed storage protein) promoter fragment of 495 base pairs, starting with 5'-TGGTCTTTTGGT-3', was placed 5' to the mutant Fad3 gene  
35 and a phaseolin termination sequence was placed 3' to the

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mutant Fad3 gene. The phaseolin sequence is described in Doyle et al., (1986) J. Biol. Chem. 261:9228-9238) and Slightom et al., (1983) Proc. Natl. Acad. Sci. USA 80:1897-1901.

5           The appropriate plasmids were engineered and transferred separately to *Agrobacterium* strain LBA4404. Each engineered strain was used to infect 5 mm segments of hypocotyl explants from Westar seeds by cocultivation. Infected hypocotyls were transferred to callus medium  
10 and, subsequently, to regeneration medium. Once discernable stems formed from the callus, shoots were excised and transferred to elongation medium. The elongated shoots were cut, dipped in Rootone™, rooted on an agar medium and transplanted to potting soil to obtain  
15 fertile T1 plants. T2 seeds were obtained by selfing the resulting T1 plants.

Fatty acid analysis of T2 seeds was carried out as described above. The results are summarized in Table XXI. Of the 40 transformants obtained using the pIMC110  
20 plasmid, 17 plants demonstrated wild type fatty acid profiles and 16 demonstrated overexpression. A proportion of the transformants are expected to display an overexpression phenotype when a functioning gene is transformed in sense orientation into plants.

25           Of the 307 transformed plants having the pIMC205 gene, none exhibited a fatty acid composition indicative of overexpression. This result indicates that the mutant fad3 gene product is non-functional, since some of the transformants would have exhibited an overexpression  
30 phenotype if the gene product were functional.

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Table XXI

Overexpression and Co-suppression Events in  
Westar Populations Transformed with pIMC205 or pIMC110.

Construct	Number of Transformants	$\alpha$ -Linolenic Acid Range(%)	Overexpression Events ( $>10\%$ linolenic)	Co-Suppression Events ( $<4.0\%$ linolenic)	Wild Type Events
pIMC110	40	2.4 - 20.6	16	7	17
pIMC205	307	4.6 - 10.4	0	0	307

Fatty acid compositions of representative transformed plants are presented in Table XXII. Lines 652-09 and 663-40 are representative of plants containing pIMC110 and exhibiting an overexpression and a co-suppression phenotype, respectively. Line 205-284 is representative of plants containing pIMC205 and having the mutant fad3 gene.

Table XXII

Fatty Acid Composition of T2 Seed  
From Westar Transformed With pIMC205 or pIMC110.

Line	Fatty Acid Composition (%)				
	C16:0	C18:0	C18:1	C18:2	C18:3
652-09 pIMC110 overexpression	4.7	3.3	65.6	8.1	14.8
663-40 pIMC110 co-suppression	4.9	2.1	62.5	23.2	3.5
205-284 pIMC205	3.7	1.8	68.8	15.9	6.7

To the extent not already indicated, it will be understood by those of ordinary skill in the art that any one of the various specific embodiments herein described and illustrated may be further modified to incorporate features shown in other of the specific embodiments.

The foregoing detailed description has been provided for a better understanding of the invention only and no unnecessary limitation should be understood therefrom as some modifications will be apparent to those



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skilled in the art without deviating from the spirit and scope of the appended claims.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Cargill, Incorporated
- (ii) TITLE OF INVENTION: PLANTS HAVING MUTANT SEQUENCES THAT CONFER  
ALTERED FATTY ACID PROFILES
- (iii) NUMBER OF SEQUENCES: 8
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Fish & Richardson, P.C., P.A.
  - (B) STREET: 60 South Sixth Street, Suite 3300
  - (C) CITY: Minneapolis
  - (D) STATE: MN
  - (E) COUNTRY: USA
  - (F) ZIP: 55402
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER: PCT/US96/
  - (B) FILING DATE: 13-DEC-1996
  - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: US 08/572,027
  - (B) FILING DATE: 14-DEC-1995
  - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Ellinger, Mark S.
  - (B) REGISTRATION NUMBER: 34,812
  - (C) REFERENCE/DOCKET NUMBER: 07148/049W01
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: 612/335-5070
  - (B) TELEFAX: 612/288-9696

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 1155 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: Brassica napus
- (ix) FEATURE:
  - (D) OTHER INFORMATION: Wild type D form.

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATG GGT GCA GGT GGA AGA ATG CAA GTG TCT CCT CCC TCC AAG AAG TCT	48
Met Gly Ala Gly Gly Arg Met Gln Val Ser Pro Pro Ser Lys Lys Ser	
1 5 10 15	
GAA ACC GAC ACC ATC AAG CGC GTA CCC TGC GAG ACA CCG CCC TTC ACT	96
Glu Thr Asp Thr Ile Lys Arg Val Pro Cys Glu Thr Pro Pro Phe Thr	
20 25 30	
GTC GGA GAA CTC AAG AAA GCA ATC CCA CCG CAC TGT TTC AAA CGC TCG	144
Val Gly Glu Leu Lys Lys Ala Ile Pro Pro His Cys Phe Lys Arg Ser	
35 40 45	
ATC CCT CGC TCT TTC TCC TAC CTC ATC TGG GAC ATC ATC ATA GCC TCC	192
Ile Pro Arg Ser Phe Ser Tyr Leu Ile Trp Asp Ile Ile Ala Ser	
50 55 60	
TGC TTC TAC TAC NTC GCC ACC ACT TAC TTC CCT CTC CTC CCT CAC CCT	240
Cys Phe Tyr Tyr Xaa Ala Thr Thr Tyr Phe Pro Leu Leu Pro His Pro	
65 70 75 80	
CTC TCC TAC TTC GCC TGG CCT CTC TAC TGG GCC TGC CAA GGG TGC GTC	288
Leu Ser Tyr Phe Ala Trp Pro Leu Tyr Trp Ala Cys Gln Gly Cys Val	
85 90 95	
CTA ACC GGC GTC TGG GTC ATA GCC CAC GAA TGC GGC CAC CAC GCC TTC	336
Leu Thr Gly Val Trp Val Ile Ala His Glu Cys Gly His His Ala Phe	
100 105 110	
AGC GAC TAC CAG TGG CTT GAC GAC ACC GTC GGT CTC ATC TTC CAC TCC	384
Ser Asp Tyr Gln Trp Leu Asp Asp Thr Val Gly Leu Ile Phe His Ser	
115 120 125	
TTC CTC CTC GTC CCT TAC TTC TCC TGG AAG TAC AGT CAT CGC AGC CAC	432
Phe Leu Leu Val Pro Tyr Phe Ser Trp Lys Tyr Ser His Arg Ser His	
130 135 140	
CAT TCC AAC ACT GGC TCC CTC GAG AGA GAC GAA GTG TTT GTC CCC AAG	480
His Ser Asn Thr Gly Ser Leu Glu Arg Asp Glu Val Phe Val Pro Lys	
145 150 155 160	
AAG AAG TCA GAC ATC AAG TGG TAC GGC AAG TAC CTC AAC AAC CCT TTG	528
Lys Lys Ser Asp Ile Lys Trp Tyr Gly Lys Tyr Leu Asn Asn Pro Leu	
165 170 175	
GGA CGC ACC GTG ATG TTA ACG GTT CAG TTC ACT CTC GGC TGG CCG TTG	576
Gly Arg Thr Val Met Leu Thr Val Gln Phe Thr Leu Gly Trp Pro Leu	
180 185 190	
TAC TTA GCC TTC AAC GTC TCG GGA AGA CCT TAC GAC GGC GGC TTC CGT	624
Tyr Leu Ala Phe Asn Val Ser Gly Arg Pro Tyr Asp Gly Gly Phe Arg	
195 200 205	
TGC CAT TTC CAC CCC AAC GCT CCC ATC TAC AAC GAC CGC GAG CGT CTC	672
Cys His Phe His Pro Asn Ala Pro Ile Tyr Asn Asp Arg Glu Arg Leu	
210 215 220	
CAG ATA TAC ATC TCC GAC GCT GGC ATC CTC GCC GTC TGC TAC GGT CTC	720
Gln Ile Tyr Ile Ser Asp Ala Gly Ile Leu Ala Val Cys Tyr Gly Leu	
225 230 235 240	
TTC CGT TAC GCC GCC GGC CAG GGA GTG GCC TCG ATG GTC TGC TTC TAC	768
Phe Arg Tyr Ala Ala Gly Gln Gly Val Ala Ser Met Val Cys Phe Tyr	
245 250 255	
GGA GTC CCG CTT CTG ATT GTC AAT GGT TTC CTC GTG TTG ATC ACT TAC	816
Gly Val Pro Leu Leu Ile Val Asn Gly Phe Leu Val Leu Ile Thr Tyr	
260 265 270	

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TTG CAG CAC ACG CAT CCT TCC CTG CCT CAC TAC GAT TCG TCC GAG TGG	864
Leu Gln His Thr His Pro Ser Leu Pro His Tyr Asp Ser Ser Glu Trp	
275 280 285	
GAT TGG TTC AGG GGA GCT TTG GCT ACC GTT GAC AGA GAC TAC GGA ATC	912
Asp Trp Phe Arg Gly Ala Leu Ala Thr Val Asp Arg Asp Tyr Gly Ile	
290 295 300	
TTG AAC AAG GTC TTC CAC AAT ATT ACC GAC ACG CAC GTG GCC CAT CAT	960
Leu Asn Lys Val Phe His Asn Ile Thr Asp Thr His Val Ala His His	
305 310 315 320	
CCG TTC TCC ACG ATG CCG CAT TAT CAC GCG ATG GAA GCT ACC AAG GCG	1008
Pro Phe Ser Thr Met Pro His Tyr His Ala Met Glu Ala Thr Lys Ala	
325 330 335	
ATA AAG CCG ATA CTG GGA GAG TAT TAT CAG TTC GAT GGG ACG CCG GTG	1056
Ile Lys Pro Ile Leu Gly Glu Tyr Tyr Gln Phe Asp Gly Thr Pro Val	
340 345 350	
GTT AAG GCG ATG TGG AGG GAG GCG AAG GAG TGT ATC TAT GTG GAA CCG	1104
Val Lys Ala Met Trp Arg Glu Ala Lys Glu Cys Ile Tyr Val Glu Pro	
355 360 365	
GAC AGG CAA GGT GAG AAG AAA GGT GTG TTC TGG TAC AAC AAT AAG TTA T	1153
Asp Arg Gln Gly Glu Lys Lys Gly Val Phe Trp Tyr Asn Asn Lys Leu	
370 375 380	
GA	1155

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 384 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Gly Ala Gly Gly Arg Met Gln Val Ser Pro Pro Ser Lys Lys Ser	
1 5 10 15	
Glu Thr Asp Thr Ile Lys Arg Val Pro Cys Glu Thr Pro Pro Phe Thr	
20 25 30	
Val Gly Glu Leu Lys Lys Ala Ile Pro Pro His Cys Phe Lys Arg Ser	
35 40 45	
Ile Pro Arg Ser Phe Ser Tyr Leu Ile Trp Asp Ile Ile Ala Ser	
50 55 60	
Cys Phe Tyr Tyr Xaa Ala Thr Thr Tyr Phe Pro Leu Leu Pro His Pro	
65 70 75 80	
Leu Ser Tyr Phe Ala Trp Pro Leu Tyr Trp Ala Cys Gln Gly Cys Val	
85 90 95	
Leu Thr Gly Val Trp Val Ile Ala His Glu Cys Gly His His Ala Phe	
100 105 110	
Ser Asp Tyr Gln Trp Leu Asp Asp Thr Val Gly Leu Ile Phe His Ser	
115 120 125	
Phe Leu Leu Val Pro Tyr Phe Ser Trp Lys Tyr Ser His Arg Ser His	
130 135 140	

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His Ser Asn Thr Gly Ser Leu Glu Arg Asp Glu Val Phe Val Pro Lys  
 145 150 155 160  
 Lys Lys Ser Asp Ile Lys Trp Tyr Gly Lys Tyr Leu Asn Asn Pro Leu  
 165 170 175  
 Gly Arg Thr Val Met Leu Thr Val Gln Phe Thr Leu Gly Trp Pro Leu  
 180 185 190  
 Tyr Leu Ala Phe Asn Val Ser Gly Arg Pro Tyr Asp Gly Gly Phe Arg  
 195 200 205  
 Cys His Phe His Pro Asn Ala Pro Ile Tyr Asn Asp Arg Glu Arg Leu  
 210 215 220  
 Gln Ile Tyr Ile Ser Asp Ala Gly Ile Leu Ala Val Cys Tyr Gly Leu  
 225 230 235 240  
 Phe Arg Tyr Ala Ala Gly Gln Gly Val Ala Ser Met Val Cys Phe Tyr  
 245 250 255  
 Gly Val Pro Leu Leu Ile Val Asn Gly Phe Leu Val Leu Ile Thr Tyr  
 260 265 270  
 Leu Gln His Thr His Pro Ser Leu Pro His Tyr Asp Ser Ser Glu Trp  
 275 280 285  
 Asp Trp Phe Arg Gly Ala Leu Ala Thr Val Asp Arg Asp Tyr Gly Ile  
 290 295 300  
 Leu Asn Lys Val Phe His Asn Ile Thr Asp Thr His Val Ala His His  
 305 310 315 320  
 Pro Phe Ser Thr Met Pro His Tyr His Ala Met Glu Ala Thr Lys Ala  
 325 330 335  
 Ile Lys Pro Ile Leu Gly Glu Tyr Tyr Gln Phe Asp Gly Thr Pro Val  
 340 345 350  
 Val Lys Ala Met Trp Arg Glu Ala Lys Glu Cys Ile Tyr Val Glu Pro  
 355 360 365  
 Asp Arg Gln Gly Glu Lys Lys Gly Val Phe Trp Tyr Asn Asn Lys Leu  
 370 375 380

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1155 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## (iii) HYPOTHETICAL: NO

## (iv) ANTI-SENSE: NO

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: Brassica napus

## (vii) IMMEDIATE SOURCE:

- (B) CLONE: IMC129

## (ix) FEATURE:

- (D) OTHER INFORMATION: G to A transversion mutation at nucleotide 316 of the D form.

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## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATG GGT GCA GGT GGA AGA ATG CAA GTG TCT CCT CCC TCC AAG AAG TCT	48
Met Gly Ala Gly Gly Arg Met Gln Val Ser Pro Pro Ser Lys Lys Ser	
1 5 10 15	
GAA ACC GAC ACC ATC AAG CGC GTA CCC TGC GAG ACA CCG CCC TTC ACT	96
Glu Thr Asp Thr Ile Lys Arg Val Pro Cys Glu Thr Pro Pro Phe Thr	
20 25 30	
GTC GGA GAA CTC AAG AAA GCA ATC CCA CCG CAC TGT TTC AAA CGC TCG	144
Val Gly Glu Leu Lys Lys Ala Ile Pro Pro His Cys Phe Lys Arg Ser	
35 40 45	
ATC CCT CGC TCT TTC TCC TAC CTC ATC TGG GAC ATC ATC ATA GCC TCC	192
Ile Pro Arg Ser Phe Ser Tyr Leu Ile Trp Asp Ile Ile Ala Ser	
50 55 60	
TGC TTC TAC TAC NTC GCC ACC ACT TAC TTC CCT CTC CTC CCT CAC CCT	240
Cys Phe Tyr Tyr Xaa Ala Thr Thr Tyr Phe Pro Leu Leu Pro His Pro	
65 70 75 80	
CTC TCC TAC TTC GCC TGG CCT CTC TAC TGG GCC TGC CAA GGG TGC GTC	288
Leu Ser Tyr Phe Ala Trp Pro Leu Tyr Trp Ala Cys Gln Gly Cys Val	
85 90 95	
CTA ACC GGC GTC TGG GTC ATA GCC CAC AAG TGC GGC CAC CAC GCC TTC	336
Leu Thr Gly Val Trp Val Ile Ala His Lys Cys Gly His His Ala Phe	
100 105 110	
AGC GAC TAC CAG TGG CTT GAC GAC ACC GTC GGT CTC ATC TTC CAC TCC	384
Ser Asp Tyr Gln Trp Leu Asp Asp Thr Val Gly Leu Ile Phe His Ser	
115 120 125	
TTC CTC CTC GTC CCT TAC TTC TCC TGG AAG TAC AGT CAT CGC AGC CAC	432
Phe Leu Leu Val Pro Tyr Phe Ser Trp Lys Tyr Ser His Arg Ser His	
130 135 140	
CAT TCC AAC ACT GGC TCC CTC GAG AGA GAC GAA GTG TTT GTC CCC AAG	480
His Ser Asn Thr Gly Ser Leu Glu Arg Asp Glu Val Phe Val Pro Lys	
145 150 155 160	
AAG AAG TCA GAC ATC AAG TGG TAC GGC AAG TAC CTC AAC AAC CCT TTG	528
Lys Lys Ser Asp Ile Lys Trp Tyr Gly Lys Tyr Leu Asn Asn Pro Leu	
165 170 175	
GGA CGC ACC GTG ATG TTA ACG GTT CAG TTC ACT CTC GGC TGG CCG TTG	576
Gly Arg Thr Val Met Leu Thr Val Gln Phe Thr Leu Gly Trp Pro Leu	
180 185 190	
TAC TTA GCC TTC AAC GTC TCG GGA AGA CCT TAC GAC GGC GGC TTC CGT	624
Tyr Leu Ala Phe Asn Val Ser Gly Arg Pro Tyr Asp Gly Gly Phe Arg	
195 200 205	
TGC CAT TTC CAC CCC AAC GCT CCC ATC TAC AAC GAC CGC GAG CGT CTC	672
Cys His Phe His Pro Asn Ala Pro Ile Tyr Asn Asp Arg Glu Arg Leu	
210 215 220	
CAG ATA TAC ATC TCC GAC GCT GGC ATC CTC GCC GTC TGC TAC GGT CTC	720
Gln Ile Tyr Ile Ser Asp Ala Gly Ile Leu Ala Val Cys Tyr Gly Leu	
225 230 235 240	
TTC CGT TAC GCC GCC GGC CAG GGA GTG GCC TCG ATG GTC TGC TTC TAC	768
Phe Arg Tyr Ala Ala Gly Gln Gly Val Ala Ser Met Val Cys Phe Tyr	
245 250 255	
GGA GTC CCG CTT CTG ATT GTC AAT GGT TTC CTC GTG TTG ATC ACT TAC	816
Gly Val Pro Leu Leu Ile Val Asn Gly Phe Leu Val Leu Ile Thr Tyr	
260 265 270	

- 69 -

TTG CAG CAC ACG CAT CCT TCC CTG CCT CAC TAC GAT TCG TCC GAG TGG	864
Leu Gln His Thr His Pro Ser Leu Pro His Tyr Asp Ser Ser Glu Trp	
275 280 285	
GAT TGG TTC AGG GGA GCT TTG GCT ACC GTT GAC AGA GAC TAC GGA ATC	912
Asp Trp Phe Arg Gly Ala Leu Ala Thr Val Asp Arg Asp Tyr Gly Ile	
290 295 300	
TTG AAC AAG GTC TTC CAC AAT ATT ACC GAC ACG CAC GTG GCC CAT CAT	960
Leu Asn Lys Val Phe His Asn Ile Thr Asp Thr His Val Ala His His	
305 310 315 320	
CCG TTC TCC ACG ATG CCG CAT TAT CAC GCG ATG GAA GCT ACC AAG GCG	1008
Pro Phe Ser Thr Met Pro His Tyr His Ala Met Glu Ala Thr Lys Ala	
325 330 335	
ATA AAG CCG ATA CTG GGA GAG TAT TAT CAG TTC GAT GGG ACG CCG GTG	1056
Ile Lys Pro Ile Leu Gly Glu Tyr Tyr Gln Phe Asp Gly Thr Pro Val	
340 345 350	
GTT AAG GCG ATG TGG AGG GAG GCG AAG GAG TGT ATC TAT GTG GAA CCG	1104
Val Lys Ala Met Trp Arg Glu Ala Lys Glu Cys Ile Tyr Val Glu Pro	
355 360 365	
GAC AGG CAA GGT GAG AAG AAA GGT GTG TTC TGG TAC AAC AAT AAG TTA T	1153
Asp Arg Gln Gly Glu Lys Lys Gly Val Phe Trp Tyr Asn Asn Lys Leu	
370 375 380	
GA	1155

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 384 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Gly Ala Gly Gly Arg Met Gln Val Ser Pro Pro Ser Lys Lys Ser	
1 5 10 15	
Glu Thr Asp Thr Ile Lys Arg Val Pro Cys Glu Thr Pro Pro Phe Thr	
20 25 30	
Val Gly Glu Leu Lys Lys Ala Ile Pro Pro His Cys Phe Lys Arg Ser	
35 40 45	
Ile Pro Arg Ser Phe Ser Tyr Leu Ile Trp Asp Ile Ile Ile Ala Ser	
50 55 60	
Cys Phe Tyr Tyr Xaa Ala Thr Thr Tyr Phe Pro Leu Leu Pro His Pro	
65 70 75 80	
Leu Ser Tyr Phe Ala Trp Pro Leu Tyr Trp Ala Cys Gln Gly Cys Val	
85 90 95	
Leu Thr Gly Val Trp Val Ile Ala His Lys Cys Gly His His Ala Phe	
100 105 110	
Ser Asp Tyr Gln Trp Leu Asp Asp Thr Val Gly Leu Ile Phe His Ser	
115 120 125	
Phe Leu Leu Val Pro Tyr Phe Ser Trp Lys Tyr Ser His Arg Ser His	
130 135 140	

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His Ser Asn Thr Gly Ser Leu Glu Arg Asp Glu Val Phe Val Pro Lys  
 145 150 155 160  
 Lys Lys Ser Asp Ile Lys Trp Tyr Gly Lys Tyr Leu Asn Asn Pro Leu  
 165 170 175  
 Gly Arg Thr Val Met Leu Thr Val Gln Phe Thr Leu Gly Trp Pro Leu  
 180 185 190  
 Tyr Leu Ala Phe Asn Val Ser Gly Arg Pro Tyr Asp Gly Gly Phe Arg  
 195 200 205  
 Cys His Phe His Pro Asn Ala Pro Ile Tyr Asn Asp Arg Glu Arg Leu  
 210 215 220  
 Gln Ile Tyr Ile Ser Asp Ala Gly Ile Leu Ala Val Cys Tyr Gly Leu  
 225 230 235 240  
 Phe Arg Tyr Ala Ala Gly Gln Gly Val Ala Ser Met Val Cys Phe Tyr  
 245 250 255  
 Gly Val Pro Leu Leu Ile Val Asn Gly Phe Leu Val Leu Ile Thr Tyr  
 260 265 270  
 Leu Gln His Thr His Pro Ser Leu Pro His Tyr Asp Ser Ser Glu Trp  
 275 280 285  
 Asp Trp Phe Arg Gly Ala Leu Ala Thr Val Asp Arg Asp Tyr Gly Ile  
 290 295 300  
 Leu Asn Lys Val Phe His Asn Ile Thr Asp Thr His Val Ala His His  
 305 310 315 320  
 Pro Phe Ser Thr Met Pro His Tyr His Ala Met Glu Ala Thr Lys Ala  
 325 330 335  
 Ile Lys Pro Ile Leu Gly Glu Tyr Tyr Gln Phe Asp Gly Thr Pro Val  
 340 345 350  
 Val Lys Ala Met Trp Arg Glu Ala Lys Glu Cys Ile Tyr Val Glu Pro  
 355 360 365  
 Asp Arg Gln Gly Glu Lys Lys Gly Val Phe Trp Tyr Asn Asn Lys Leu  
 370 375 380

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1155 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## (iii) HYPOTHETICAL: NO

## (iv) ANTI-SENSE: NO

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: Brassica napus

## (ix) FEATURE:

- (D) OTHER INFORMATION: Wild type F form.



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## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATG Met 1	GGT Gly 5	GCA Ala 10	GGT Gly 15	GGA Gly 20	AGA Arg 25	ATG Met 30	CAA Gln 35	GTG Val 40	TCT Ser 45	CCT Pro 50	CCC Pro 55	TCC Ser 60	AAA Lys 65	AAG Lys 70	TCT Ser 75	48
GAA Glu 20	ACC Thr 25	GAC Asp 30	AAC Asn 35	ATC Ile 40	AAG Lys 45	CGC Arg 50	GTA Val 55	CCC Pro 60	TGC Cys 65	GAG Glu 70	ACA Thr 75	CCG Pro 80	CCC Pro 85	TTC Phe 90	ACT Thr 95	96
GTC Val 35	GGA Gly 40	GAA Glu 45	CTC Leu 50	AAG Lys 55	AAA Lys 60	GCA Ala 65	ATC Ile 70	CCA Pro 75	CCG Pro 80	CAC His 85	TGT Cys 90	TTC Phe 95	AAA Lys 100	CGC Arg 105	TCG Ser 110	144
ATC Ile 50	CCT Pro 55	CGC Arg 60	TCT Ser 65	TTC Phe 70	TCC Ser 75	TAC Tyr 80	CTC Leu 85	ATC Ile 90	TGG Trp 95	GAC Asp 100	ATC Ile 105	ATC Ile 110	ATA Ile 115	GCC Ala 120	TCC Ser 125	192
TGC Cys 65	TTC Phe 70	TAC Tyr 75	TAC Tyr 80	GTC Val 85	GCC Ala 90	ACC Thr 95	ACT Thr 100	TAC Tyr 105	TTC Phe 110	CCT Pro 115	CTC Leu 120	CTC Leu 125	CCT Pro 130	CAC His 135	CCT Pro 140	240
CTC Leu 85	TCC Ser 90	TAC Tyr 95	TTC Phe 100	GCC Ala 105	TGG Trp 110	CCT Pro 115	CTC Leu 120	TAC Tyr 125	TGG Trp 130	GCC Ala 135	TGC Cys 140	CAG Gln 145	GGC Gly 150	TGC Cys 155	GTC Val 160	288
CTA Leu 100	ACC Thr 105	GGC Gly 110	GTC Val 115	TGG Trp 120	GTC Val 125	ATA Ile 130	GCC Ala 135	CAC His 140	GAG Glu 145	TGC Cys 150	GGC Gly 155	CAC His 160	CAC His 165	GCC Ala 170	TTC Phe 175	336
AGC Ser 115	GAC Asp 120	TAC Tyr 125	CAG Gln 130	TGG Trp 135	CTG Leu 140	GAC Asp 145	GAC Asp 150	ACC Thr 155	GTC Val 160	GGC Gly 165	CTC Leu 170	ATC Ile 175	TTC Phe 180	CAC His 185	TCC Ser 190	384
TTC Phe 130	CTC Leu 135	CTC Leu 140	GTC Val 145	CCT Pro 150	TAC Tyr 155	TTC Phe 160	TCC Ser 165	TGG Trp 170	AAG Lys 175	TAC Tyr 180	AGT Ser 185	CAT His 190	CGA Arg 195	CGC Arg 200	CAC His 205	432
CAT His 145	TCC Ser 150	AAC Asn 155	ACT Thr 160	GGC Gly 165	TCC Ser 170	CTC Leu 175	GAG Glu 180	AGA Arg 185	GAC Asp 190	GAA Glu 195	GTG Val 200	TTT Phe 205	GTC Val 210	CCC Pro 215	AAG Lys 220	480
AAG Lys 165	AAG Lys 170	TCA Ser 175	GAC Asp 180	ATC Ile 185	AAG Lys 190	TGG Trp 195	TAC Tyr 200	GGC Gly 205	AAG Lys 210	TAC Tyr 215	CTC Leu 220	AAC Asn 225	AAC Asn 230	CCT Pro 235	TTG Leu 240	528
GGA Gly 180	CGC Arg 185	ACC Thr 190	GTG Val 195	ATG Met 200	TTA Leu 205	ACG Thr 210	GTT Val 215	CAG Gln 220	TTC Phe 225	ACT Thr 230	CTC Leu 235	GGC Gly 240	TGG Trp 245	CCT Pro 250	TTG Leu 255	576
TAC Tyr 195	TTA Leu 200	GCC Ala 205	TTC Phe 210	AAC Asn 215	GTC Val 220	TCG Ser 225	GGG Gly 230	AGA Arg 235	CCT Pro 240	TAC Tyr 245	GAC Asp 250	GGC Gly 255	GGC Gly 260	TTC Phe 265	GCT Ala 270	624
TGC Cys 210	CAT His 215	TTC Phe 220	CAC His 225	CCC Pro 230	AAC Asn 235	GCT Ala 240	CCC Pro 245	ATC Ile 250	TAC Tyr 255	AAC Asn 260	GAC Asp 265	CGC Arg 270	GAG Glu 275	CGT Arg 280	CTC Leu 285	672
CAG Gln 225	ATA Ile 230	TAC Tyr 235	ATC Ile 240	TCC Ser 245	GAC Asp 250	GCT Ala 255	GGC Gly 260	ATC Ile 265	CTC Leu 270	GCC Ala 275	GTC Val 280	TGC Cys 285	TAC Tyr 290	GGT Gly 295	CTC Leu 300	720
TAC Tyr 245	CGC Arg 250	TAC Tyr 255	GCT Ala 260	GCT Ala 265	GTC Val 270	CAA Gln 275	GGA Gly 280	GTT Val 285	GCC Ala 290	TCG Ser 295	ATG Met 300	GTC Val 305	TGC Cys 310	TTC Phe 315	TAC Tyr 320	768
GGA Gly 260	GTT Val 265	CCG Pro 270	CTT Leu 275	CTG Leu 280	ATT Ile 285	GTC Val 290	AAT Asn 295	GGG Gly 300	TTC Phe 305	TTA Leu 310	GTT Val 315	TTG Leu 320	ATC Ile 325	ACT Thr 330	TAC Tyr 335	816

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TTG CAG CAC ACG CAT CCT TCC CTG CCT CAC TAT GAC TCG TCT GAG TGG	864
Leu Gln His Thr His Pro Ser Leu Pro His Tyr Asp Ser Ser Glu Trp	
275 280 285	
GAT TGG TTG AGG GGA GCT TTG GCC ACC GTT GAC AGA GAC TAC GGA ATC	912
Asp Trp Leu Arg Gly Ala Leu Ala Thr Val Asp Arg Asp Tyr Gly Ile	
290 295 300	
TTG AAC AAG GTC TTC CAC AAT ATC ACG GAC ACG CAC GTG GCG CAT CAC	960
Leu Asn Lys Val Phe His Asn Ile Thr Asp Thr His Val Ala His His	
305 310 315 320	
CTG TTC TCG ACC ATG CCG CAT TAT CAT GCG ATG GAA GCT ACG AAG GCG	1008
Leu Phe Ser Thr Met Pro His Tyr His Ala Met Glu Ala Thr Lys Ala	
325 330 335	
ATA AAG CCG ATA CTG GGA GAG TAT TAT CAG TTG CAT GGG ACG CCG GTG	1056
Ile Lys Pro Ile Leu Gly Glu Tyr Tyr Gln Leu His Gly Thr Pro Val	
340 345 350	
GTT AAG GCG ATG TGG AGG GAG GCG AAG GAG TGT ATC TAT GTG GAA CCG	1104
Val Lys Ala Met Trp Arg Glu Ala Lys Glu Cys Ile Tyr Val Glu Pro	
355 360 365	
GAC AGG CAA GGT GAG AAG AAA GGT GTG TTC TGG TAC AAC AAT AAG TTA T	1153
Asp Arg Gln Gly Glu Lys Lys Gly Val Phe Trp Tyr Asn Asn Lys Leu	
370 375 380	
GA	1155

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 384 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Gly Ala Gly Gly Arg Met Gln Val Ser Pro Pro Ser Lys Lys Ser	
1 5 10 15	
Glu Thr Asp Asn Ile Lys Arg Val Pro Cys Glu Thr Pro Pro Phe Thr	
20 25 30	
Val Gly Glu Leu Lys Lys Ala Ile Pro Pro His Cys Phe Lys Arg Ser	
35 40 45	
Ile Pro Arg Ser Phe Ser Tyr Leu Ile Trp Asp Ile Ile Ala Ser	
50 55 60	
Cys Phe Tyr Tyr Val Ala Thr Thr Tyr Phe Pro Leu Leu Pro His Pro	
65 70 75 80	
Leu Ser Tyr Phe Ala Trp Pro Leu Tyr Trp Ala Cys Gln Gly Cys Val	
85 90 95	
Leu Thr Gly Val Trp Val Ile Ala His Glu Cys Gly His His Ala Phe	
100 105 110	
Ser Asp Tyr Gln Trp Leu Asp Asp Thr Val Gly Leu Ile Phe His Ser	
115 120 125	
Phe Leu Leu Val Pro Tyr Phe Ser Trp Lys Tyr Ser His Arg Arg His	
130 135 140	

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His Ser Asn Thr Gly Ser Leu Glu Arg Asp Glu Val Phe Val Pro Lys
145                      150                      155                      160

Lys Lys Ser Asp Ile Lys Trp Tyr Gly Lys Tyr Leu Asn Asn Pro Leu
                      165                      170                      175

Gly Arg Thr Val Met Leu Thr Val Gln Phe Thr Leu Gly Trp Pro Leu
                      180                      185                      190

Tyr Leu Ala Phe Asn Val Ser Gly Arg Pro Tyr Asp Gly Gly Phe Ala
                      195                      200                      205

Cys His Phe His Pro Asn Ala Pro Ile Tyr Asn Asp Arg Glu Arg Leu
210                      215                      220

Gln Ile Tyr Ile Ser Asp Ala Gly Ile Leu Ala Val Cys Tyr Gly Leu
225                      230                      235                      240

Tyr Arg Tyr Ala Ala Val Gln Gly Val Ala Ser Met Val Cys Phe Tyr
                      245                      250                      255

Gly Val Pro Leu Leu Ile Val Asn Gly Phe Leu Val Leu Ile Thr Tyr
                      260                      265                      270

Leu Gln His Thr His Pro Ser Leu Pro His Tyr Asp Ser Ser Glu Trp
275                      280                      285

Asp Trp Leu Arg Gly Ala Leu Ala Thr Val Asp Arg Asp Tyr Gly Ile
290                      295                      300

Leu Asn Lys Val Phe His Asn Ile Thr Asp Thr His Val Ala His His
305                      310                      315                      320

Leu Phe Ser Thr Met Pro His Tyr His Ala Met Glu Ala Thr Lys Ala
                      325                      330                      335

Ile Lys Pro Ile Leu Gly Glu Tyr Tyr Gln Leu His Gly Thr Pro Val
                      340                      345                      350

Val Lys Ala Met Trp Arg Glu Ala Lys Glu Cys Ile Tyr Val Glu Pro
355                      360                      365

Asp Arg Gln Gly Glu Lys Lys Gly Val Phe Trp Tyr Asn Asn Lys Leu
370                      375                      380

```

## (2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1155 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:  
 (A) ORGANISM: Brassica napus

(vii) IMMEDIATE SOURCE:  
 (B) CLONE: Q508

(ix) FEATURE:

(D) OTHER INFORMATION: T to A transversion  
 mutation at nucleotide 515 of the F form.

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## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATG GGT GCA GGT GGA AGA ATG CAA GTG TCT CCT CCC TCC AAA AAG TCT	48
Met Gly Ala Gly Gly Arg Met Gln Val Ser Pro Pro Ser Lys Lys Ser	
1 5 10 15	
GAA ACC GAC AAC ATC AAG CGC GTA CCC TGC GAG ACA CCG CCC TTC ACT	96
Glu Thr Asp Asn Ile Lys Arg Val Pro Cys Glu Thr Pro Pro Phe Thr	
20 25 30	
GTC GGA GAA CTC AAG AAA GCA ATC CCA CCG CAC TGT TTC AAA CGC TCG	144
Val Gly Glu Leu Lys Lys Ala Ile Pro Pro His Cys Phe Lys Arg Ser	
35 40 45	
ATC CCT CGC TCT TTC TCC TAC CTC ATC TGG GAC ATC ATC ATA GCC TCC	192
Ile Pro Arg Ser Phe Ser Tyr Leu Ile Trp Asp Ile Ile Ile Ala Ser	
50 55 60	
TGC TTC TAC TAC GTC GCC ACC ACT TAC TTC CCT CTC CTC CCT CAC CCT	240
Cys Phe Tyr Tyr Val Ala Thr Thr Tyr Phe Pro Leu Leu Pro His Pro	
65 70 75 80	
CTC TCC TAC TTC GCC TGG CCT CTC TAC TGG GCC TGC CAG GGC TGC GTC	288
Leu Ser Tyr Phe Ala Trp Pro Leu Tyr Trp Ala Cys Gln Gly Cys Val	
85 90 95	
CTA ACC GGC GTC TGG GTC ATA GCC CAC GAG TGC GGC CAC CAC GCC TTC	336
Leu Thr Gly Val Trp Val Ile Ala His Glu Cys Gly His His Ala Phe	
100 105 110	
AGC GAC TAC CAG TGG CTG GAC GAC ACC GTC GGC CTC ATC TTC CAC TCC	384
Ser Asp Tyr Gln Trp Leu Asp Asp Thr Val Gly Leu Ile Phe His Ser	
115 120 125	
TTC CTC CTC GTC CCT TAC TTC TCC TGG AAG TAC AGT CAT CGA CGC CAC	432
Phe Leu Leu Val Pro Tyr Phe Ser Trp Lys Tyr Ser His Arg Arg His	
130 135 140	
CAT TCC AAC ACT GGC TCC CTC GAG AGA GAC GAA GTG TTT GTC CCC AAG	480
His Ser Asn Thr Gly Ser Leu Glu Arg Asp Glu Val Phe Val Pro Lys	
145 150 155 160	
AAG AAG TCA GAC ATC AAG TGG TAC GGC AAG TAC CAC AAC AAC CCT TTG	528
Lys Lys Ser Asp Ile Lys Trp Tyr Gly Lys Tyr His Asn Asn Pro Leu	
165 170 175	
GGA CGC ACC GTG ATG TTA ACG GTT CAG TTC ACT CTC GGC TGG CCT TTG	576
Gly Arg Thr Val Met Leu Thr Val Gln Phe Thr Leu Gly Trp Pro Leu	
180 185 190	
TAC TTA GCC TTC AAC GTC TCG GGG AGA CCT TAC GAC GGC GGC TTC GCT	624
Tyr Leu Ala Phe Asn Val Ser Gly Arg Pro Tyr Asp Gly Gly Phe Ala	
195 200 205	
TGC CAT TTC CAC CCC AAC GCT CCC ATC TAC AAC GAC CGC GAG CGT CTC	672
Cys His Phe His Pro Asn Ala Pro Ile Tyr Asn Asp Arg Glu Arg Leu	
210 215 220	
CAG ATA TAC ATC TCC GAC GCT GGC ATC CTC GCC GTC TGC TAC GGT CTC	720
Gln Ile Tyr Ile Ser Asp Ala Gly Ile Leu Ala Val Cys Tyr Gly Leu	
225 230 235 240	
TAC CGC TAC GCT GCT GTC CAA GGA GTT GCC TCG ATG GTC TGC TTC TAC	768
Tyr Arg Tyr Ala Val Gln Gly Val Ala Ser Met Val Cys Phe Tyr	
245 250 255	
GGA GTT CCG CTT CTG ATT GTC AAT GGG TTC TTA GTT TTG ATC ACT TAC	816
Gly Val Pro Leu Leu Ile Val Asn Gly Phe Leu Val Leu Ile Thr Tyr	
260 265 270	

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TTG CAG CAC ACG CAT CCT TCC CTG CCT CAC TAT GAC TCG TCT GAG TGG	864
Leu Gln His Thr His Pro Ser Leu Pro His Tyr Asp Ser Ser Glu Trp	
275 280 285	
GAT TGG TTG AGG GGA GCT TTG GCC ACC GTT GAC AGA GAC TAC GGA ATC	912
Asp Trp Leu Arg Gly Ala Leu Ala Thr Val Asp Arg Asp Tyr Gly Ile	
290 295 300	
TTG AAC AAG GTC TTC CAC AAT ATC ACG GAC ACG CAC GTG GCG CAT CAC	960
Leu Asn Lys Val Phe His Asn Ile Thr Asp Thr His Val Ala His His	
305 310 315 320	
CTG TTC TCG ACC ATG CCG CAT TAT CAT GCG ATG GAA GCT ACG AAG GCG	1008
Leu Phe Ser Thr Met Pro His Tyr His Ala Met Glu Ala Thr Lys Ala	
325 330 335	
ATA AAG CCG ATA CTG GGA GAG TAT TAT CAG TTG CAT GGG ACG CCG GTG	1056
Ile Lys Pro Ile Leu Gly Glu Tyr Tyr Gln Leu His Gly Thr Pro Val	
340 345 350	
GTT AAG GCG ATG TGG AGG GAG GCG AAG GAG TGT ATC TAT GTG GAA CCG	1104
Val Lys Ala Met Trp Arg Glu Ala Lys Glu Cys Ile Tyr Val Glu Pro	
355 360 365	
GAC AGG CAA GGT GAG AAG AAA GGT GTG TTC TGG TAC AAC AAT AAG TTA T	1153
Asp Arg Gln Gly Glu Lys Lys Gly Val Phe Trp Tyr Asn Asn Lys Leu	
370 375 380	
GA	1155

## (2) INFORMATION FOR SEQ ID NO:8:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 384 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met Gly Ala Gly Gly Arg Met Gln Val Ser Pro Pro Ser Lys Lys Ser	
1 5 10 15	
Glu Thr Asp Asn Ile Lys Arg Val Pro Cys Glu Thr Pro Pro Phe Thr	
20 25 30	
Val Gly Glu Leu Lys Lys Ala Ile Pro Pro His Cys Phe Lys Arg Ser	
35 40 45	
Ile Pro Arg Ser Phe Ser Tyr Leu Ile Trp Asp Ile Ile Ile Ala Ser	
50 55 60	
Cys Phe Tyr Tyr Val Ala Thr Thr Tyr Phe Pro Leu Leu Pro His Pro	
65 70 75 80	
Leu Ser Tyr Phe Ala Trp Pro Leu Tyr Trp Ala Cys Gln Gly Cys Val	
85 90 95	
Leu Thr Gly Val Trp Val Ile Ala His Glu Cys Gly His His Ala Phe	
100 105 110	
Ser Asp Tyr Gln Trp Leu Asp Asp Thr Val Gly Leu Ile Phe His Ser	
115 120 125	
Phe Leu Leu Val Pro Tyr Phe Ser Trp Lys Tyr Ser His Arg Arg His	
130 135 140	

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His Ser Asn Thr Gly Ser Leu Glu Arg Asp Glu Val Phe Val Pro Lys  
 145 150 155 160  
 Lys Lys Ser Asp Ile Lys Trp Tyr Gly Lys Tyr His Asn Asn Pro Leu  
 165 170 175  
 Gly Arg Thr Val Met Leu Thr Val Gln Phe Thr Leu Gly Trp Pro Leu  
 180 185 190  
 Tyr Leu Ala Phe Asn Val Ser Gly Arg Pro Tyr Asp Gly Gly Phe Ala  
 195 200 205  
 Cys His Phe His Pro Asn Ala Pro Ile Tyr Asn Asp Arg Glu Arg Leu  
 210 215 220  
 Gln Ile Tyr Ile Ser Asp Ala Gly Ile Leu Ala Val Cys Tyr Gly Leu  
 225 230 235 240  
 Tyr Arg Tyr Ala Ala Val Gln Gly Val Ala Ser Met Val Cys Phe Tyr  
 245 250 255  
 Gly Val Pro Leu Leu Ile Val Asn Gly Phe Leu Val Leu Ile Thr Tyr  
 260 265 270  
 Leu Gln His Thr His Pro Ser Leu Pro His Tyr Asp Ser Ser Glu Trp  
 275 280 285  
 Asp Trp Leu Arg Gly Ala Leu Ala Thr Val Asp Arg Asp Tyr Gly Ile  
 290 295 300  
 Leu Asn Lys Val Phe His Asn Ile Thr Asp Thr His Val Ala His His  
 305 310 315 320  
 Leu Phe Ser Thr Met Pro His Tyr His Ala Met Glu Ala Thr Lys Ala  
 325 330 335  
 Ile Lys Pro Ile Leu Gly Glu Tyr Tyr Gln Leu His Gly Thr Pro Val  
 340 345 350  
 Val Lys Ala Met Trp Arg Glu Ala Lys Glu Cys Ile Tyr Val Glu Pro  
 355 360 365  
 Asp Arg Gln Gly Glu Lys Lys Gly Val Phe Trp Tyr Asn Asn Lys Leu  
 370 375 380

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## WHAT IS CLAIMED IS:

1. An isolated nucleic acid fragment comprising a sequence of at least about 10 nucleotides from a *Brassicaceae* or *Helianthus* delta-12 fatty acid desaturase gene having at least one mutation, wherein said gene is effective for altering fatty acid composition in *Brassicaceae* or *Helianthus* seeds and wherein said sequence includes said at least one mutation.
2. The nucleic acid fragment of claim 1, wherein said sequence comprises a full-length coding sequence of said gene.
3. The nucleic acid fragment of claim 1, wherein said mutant desaturase gene encodes a microsomal gene product.
4. The nucleic acid fragment of claim 1, wherein said at least one mutation comprises a mutation in a region of said desaturase gene encoding a His-Glu-Cys-Gly-His amino acid motif.
5. The nucleic acid fragment of claim 4, wherein said at least one mutation comprises a non-conservative amino acid substitution in said region.
6. The nucleic acid fragment of claim 5, wherein said at least one mutation comprises the sequence His-Lys-Cys-Gly-His.
7. The nucleic acid fragment of claim 1, wherein said mutant desaturase gene is from a *Brassica napus* plant.
8. The nucleic acid fragment of claim 1, wherein said gene is the D form of a *Brassicaceae* microsomal gene.

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9. The nucleic acid fragment of claim 1, wherein said at least at least one mutation comprises the sequence Lys-Tyr-His-Asn-Asn-Pro.

10. A plant of the *Brassicaceae* or *Helianthus* families  
5 other than *Brassica napus*, said plant containing a sequence of at least 10 nucleotides from a delta-12 fatty acid desaturase gene having at least one mutation, said at least one mutation in a region encoding a His-Xaa-Xaa-Xaa-His amino acid motif and wherein said mutation  
10 confers an altered fatty acid composition in seeds of said plant.

11. The plant of claim 10, wherein said plant contains a full-length coding sequence of said mutant gene.

12. The plant of claim 10, wherein said motif  
15 comprises the sequence His-Glu-Cys-Gly-His.

13. The plant of claim 10, wherein said gene is from a *Brassica napus* plant.

14. The plant of claim 10, wherein said plant is a *Brassica rapa* plant.

20 15. An isolated nucleic acid fragment comprising a sequence of at least about 10 nucleotides from a *Brassicaceae* or *Helianthus* delta-15 fatty acid desaturase gene having at least one mutation, wherein said gene is effective for altering fatty acid composition in  
25 *Brassicaceae* or *Helianthus* seeds and wherein said sequence includes said at least one mutation.



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16. The nucleic acid fragment of claim 15, wherein said sequence comprises a full-length coding sequence of said gene.
17. The nucleic acid fragment of claim 15, wherein said at least one mutation comprises a mutation in a region of said desaturase gene encoding a His-Asp-Cys-Gly-His amino acid motif.
18. The nucleic acid fragment of claim 15, wherein said mutant desaturase gene is from a *Brassica napus* plant.
19. A Brassicaceae or *Helianthus* plant containing a sequence of at least 10 nucleotides from a delta-15 fatty acid desaturase gene having at least one mutation, said at least one mutation in a region encoding a His-Xaa-Xaa-Xaa-His amino acid motif and wherein said mutation confers an altered fatty acid composition in seeds of said plant.
20. The plant of claim 19, wherein said plant contains a full-length coding sequence of said mutant gene.
21. The plant of claim 19, wherein said motif comprises the sequence His-Asp-Cys-Gly-His.
22. The plant of claim 19, wherein said mutant desaturase gene is from a *Brassica napus* plant.
23. The plant of claim 19, wherein said plant is a *Brassica napus* plant.
24. A Brassicaceae or *Helianthus* plant containing:

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- a) a sequence of at least about 10 nucleotides from a delta-12 fatty acid desaturase gene having at least one mutation, said at least one delta-12 gene mutation in a region encoding a His-Xaa-Xaa-Xaa-His amino acid motif;
- b) a sequence of at least 10 nucleotides from a delta-15 fatty acid desaturase gene having at least one mutation, said at least one delta-15 gene mutation in a region encoding a His-Xaa-Xaa-Xaa-His amino acid motif, said delta-12 gene mutation and said delta-15 gene mutation conferring an altered fatty acid composition in seeds of said plant.

25. A Brassicaceae or Helianthus plant containing a sequence of at least about 10 nucleotides from a delta-12 fatty acid desaturase gene having at least one mutation, said at least one mutation in a region encoding a Tyr-Leu-Asn-Asn-Pro amino acid motif and wherein said mutation confers an altered fatty acid composition in seeds of said plant.

26. A vegetable oil extracted from seeds produced by the plant of claim 10.

27. The oil of claim 26, wherein said oil has, following crushing and extraction of said seeds, from about 1% to about 10% linoleic acid based on total fatty acid composition.

28. The oil of claim 26, wherein said oil has from about 69% to about 90% oleic acid based on total fatty acid composition.

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29. A vegetable oil extracted from seeds produced by the plant of claim 19.

30. The oil of claim 29, wherein said oil has, following crushing and extraction of said seeds, from  
5 about 0.5% to about 10%  $\alpha$ -linolenic acid based on total fatty acid composition.

31. A vegetable oil extracted from seeds produced by the plant of claim 24.

32. A vegetable oil extracted from seeds produced by  
10 the plant of claim 25.

33. A method for producing a *Brassicaceae* or *Helianthus* plant line, comprising the steps of:

- a) inducing mutagenesis in cells of a starting variety of a *Brassicaceae* or *Helianthus* species;
- 15 b) obtaining one or more progeny plants from said cells;
- c) identifying at least one of said progeny plant that contains a delta-12 fatty acid desaturase gene having at least one mutation, said  
20 at least one mutation in a region encoding a His-Xaa-Xaa-Xaa-His amino acid motif; and
- d) producing said plant line from said at least one progeny plant by self- or cross-pollination, said plant line having said at least one delta-12  
25 gene mutation.

34. The method of claim 33, wherein said plant line produces seeds yielding an oil having a stabilized linoleic acid content from about 1% to about 14%.

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35. The method of claim 33, further comprising the steps of:

- e) inducing mutagenesis in cells of said plant line;
- 5 f) obtaining one or more progeny plants from said plant line cells;
- g) identifying at least one of said plant line progeny plants that contains a delta-15 fatty acid desaturase gene having at least one delta-15 gene mutation, said at least one delta-15 gene mutation  
10 in a region encoding a His-Xaa-Xaa-Xaa-His amino acid motif;
- h) producing a second plant line from said at least one plant line progeny plant by self- or  
15 cross-pollination, said second plant line having said at least one delta-12 gene mutation and said at least one delta-15 gene mutation.

36. The method of claim 33, wherein said starting variety is a *Brassica napus* variety.

20 37. The method of claim 36, wherein said mutation is in a first form of delta-12 fatty acid desaturase.

38. The method of claim 37, further comprising the step of crossing a plant of said plant line to a plant having a mutation in a second form of delta-12 fatty acid  
25 desaturase.

39. The method of claim 38, wherein said second mutation is in a region other than a region encoding a His-Xaa-Xaa-Xaa-His amino acid motif.

40. The method of claim 36, further comprising the  
30 steps of:

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- e) inducing mutagenesis in cells of said plant line;
- f) obtaining one or more progeny plants from said plant line cells;
- 5 g) identifying at least one of said plant line progeny plants that contains a second delta-12 fatty acid desaturase gene having at least one mutation, said second gene mutation in a region other than a region encoding a His-Xaa-Xaa-Xaa-His amino acid motif; and
- 10 h) producing a second plant line from said at least one plant line progeny plant by self- or cross-pollination, said second plant line having said first delta-12 gene mutation and said second
- 15 delta-12 gene mutation.

41. A method for producing a *Brassicaceae* or *Helianthus* plant line, comprising the steps of:
- a) inducing mutagenesis in cells of a starting variety of a *Brassicaceae* or *Helianthus* species;
  - 20 b) obtaining one or more progeny plants from said cells;
  - c) identifying at least one of said progeny plants that contains a delta-15 fatty acid desaturase gene having at least one mutation, said
  - 25 at least one mutation in a region encoding a His-Xaa-Xaa-Xaa-His amino acid motif; and
  - d) producing said plant line from said at least one progeny plant by self- or cross-pollination, said plant line having said delta-15 gene
  - 30 mutation.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/20090

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : A01H 1/06, 5/10, 1/00; C12N 15/00; C07C 57/02, 57/03, 53/126

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/230, 200, 205, 255, DIG. 17, DIG 69; 435/172.1, 172.3; 47/58, DIG. 1; 554/8, 9, 223, 224; 426/601, 615, 629

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ROBBELEN. Genetical and Physiological Investigations on Mutants for Polyenoic Fatty Acids in Rapeseed, Brassica napus L. Z. Pflanzenzuchtg. 1975. Vol. 73, pages 93-105, especially page 94.	10-14 and 19-41
Y	WO 91/15578 A1 (PIONEER HI-BRED INTERNATIONAL, INC.) 17 October 1991, pages 1-56, especially pages 8-40.	10-41
Y	US 5,434,283 A (WONG et al.) 18 June 1995, columns 1-20, especially column 4, line 50 to column 19, line 8.	10-41
Y	EP O 323 753 A1 (ALLELIX INC.) 12 July 1989, pages 2-12, especially pages 7-10.	10-41



Further documents are listed in the continuation of Box C.



See patent family annex.

- \* Special categories of cited documents:
- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- \*Z\* document member of the same patent family

Date of the actual completion of the international search

20 MARCH 1997

Date of mailing of the international search report

08 MAY 1997

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
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Washington, D.C. 20231

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Authorized officer

GARY BENZION

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)\*

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/20090

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TOPFER et al. Modification of Plant Lipid Synthesis. SCIENCE, Vol. 268, 05 May 1995, pages 681-686.	1-9, 10-14, 19-25 and 33-41
Y	SCARTH et al. STELLAR LOW LINOLENIC -HIGH LINOLEIC ACID SUMMER RAPE. Can. J. Plant Sci. Apr. 1988, Vol. 68, pages 509-511.	10-14, 19-25 and 26-32
Y	US 4,948,811 A (SPINNER et al.) 14 August 1990, columns 1-8.	26-32
Y	US 5,387,758 A (WONG et al.) 07 February 1995, columns 2-24, especially column 11, line 25 to column 24, line 26.	10-41
Y	WO 93/12245 A1 (E.I. DU PONT DE NEMOURS AND COMPANY) 10 June 1993, pages 1-163, especially pages 25 to 85.	1-41

Form PCT/ISA/210 (continuation of second sheet)(July 1992)\*

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/20090

## A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

800/230, 200, 205, 255, DIG. 17, DIG 69; 435/172.1, 172.3; 47/58, DIG. 1; 554/8, 9, 223, 224; 426/601, 615, 629

## B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

## APS, BIOSIS, DIALOG.

search terms: nucleic acid, delta 12 fatty acid desaturase, delta 9 fatty acid desaturase, Brassica napus, Brassicaceae, Helianthus, mutagenesis, mutation breeding, linoleic, oleic, alpha linolenic.

Form PCT/ISA/210 (extra sheet)(July 1992)\*